University of Alberta

Maturation of Fatty Acid Oxidation in the Newborn Rabbit Heart

by

Abiola Olufemi Makinde ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

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Dedication

To my wife
and the boys

“You still love me in spite of my long hours away from home in the lab”

Abstract

Fatty acid oxidation dramatically increases secondary to a decreased acetyl CoA carboxylase (ACC) activity in 7-day compared to 1-day old rabbit hearts. The mechanism responsible for this rapid maturation of fatty acid oxidation and decreased ACC activity remain poorly understood. I hypothesized that increased 5'AMP-activated protein kinase (AMPK) activity is responsible for the decreased ACC activity in 7-day old hearts. To test this hypothesis, hearts from 1-day and 7-day old rabbits were used to characterize AMPK activity and abundance. Immunoblot analysis revealed that AMPK is present in both 1-day and 7-day old hearts. AMPK abundance was significantly upregulated in 7-day compared to 1-day old heart. Moreover, AMPK activity measured in Langendorff perfused hearts increased from 510 ± 47 to 792 ± 38 pmol / min / mg protein ($p < 0.05$) in 1-day and 7-day old hearts, respectively. This was paralleled by a decrease in ACC activity in 7-day compared to 1-day old hearts. Furthermore, fatty acid oxidation increased from 14 ± 3 to 32 ± 5 nmol / min / g dry ($p < 0.05$), in 1 day and 7-day old hearts, respectively.

AICAR (200 μM), a cell permeable nucleoside, stimulated AMPK activity and fatty acid oxidation in 7-day old hearts. Iodotubercidin (50 μM), an AMPK inhibitor, however, decreased fatty acid oxidation compared to controls. AMPK activity was positively correlated with fatty acid oxidation. Moreover, in reperfused-ischemic hearts, iodotubercidin (50 μM) significantly decreased AMPK activity and fatty acid oxidation compared to controls. In addition, hearts treated with iodotubercidin showed a significant improvement in functional

recovery [92 ± 4 vs 52 ± 3%, ($p<0.05$) in iodotubercidin and control hearts, respectively] during reperfusion.

In summary this study demonstrates that AMPK is important in the maturation and regulation of fatty acid oxidation in newborn hearts. Moreover, AMPK inhibitors improved functional recovery during reperfusion following ischemia in 7-day old hearts. If AMPK inhibitors improve functional recovery in the clinical setting of myocardial ischemia, they may be novel therapeutics in the treatment of ischemic heart disease.

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Table of Contents

Abstract

Acknowledgements

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Chapter — Page

I. Introduction 1

Background 2

Fatty Acid Uptake and Metabolism 5

Regulation of Fatty Acid Metabolism in the Heart 7

Purpose of This Study 21

2. General Experimental Methods and Materials 45

Heart Perfusions 47

Determination of Palmitate Oxidation Rates 50

AMP-activated Protein Kinase Assay 53

Acetyl CoA Carboxylase Assay 54

Determination of CoA esters Levels 59

References 67

3. Characterization of the 5'AMP-activated Protein Kinase in Both Fetal and Newborn Rabbit Hearts 70

Introduction 71

Experimental Procedures 73

Results 78
Discussion 82
References 120
4. Upregulation of 5'AMP activated Protein Kinase is Responsible for the Increase in Myocardial Fatty Acid Oxidation Following Birth in Newborn Rabbits 126
Introduction 127
Experimental Procedures 130
Results 133
Discussion 137
References 158
5. Evidence that 5'AMP-activated Protein Kinase Activity Regulates Myocardial Fatty Acid Oxidation in Newborn Rabbit Hearts 163
Introduction 164
Experimental Procedures 166
Results 170
Discussion 177
References 203
6 5'AMP-activated Protein Kinase Inhibitors Improve the Recovery of Mechanical Function Following Ischemia in Newborn Rabbit Hearts 208
Introduction 209
Experimental Procedures 211
Results 215

Discussion 220

References 247

7. Glucagon and Isoproterenol Stimulate Fatty Acid Oxidation in Newborn Rabbit Hearts by 5'AMP activated Protein Kinase Independent Mechanism. 252

Introduction 253

Experimental Procedures 255

Results 258

Discussion 261

References 281

8. General Discussion and Conclusions 285

Future Direction 306

References 311

List of Tables

Tables	Description	Page
1-1	Overview of changes in circulating substrate and hormone levels in the fetus, 1 week-old and adult experimental animals.	26
1-2	Overview of normal contribution of energy substrates to total ATP production in the fetal, 1-day old, 1 week-old, and adult hearts.	27
3-1	Selected studies of AMP-activated protein kinase in various tissues and cell cultures.	88
3-2	AMPK activity measured in PEG 8000 extracts from unperfused 7-day old rabbit hearts using SAMS peptide or AMARA peptide as the synthetic peptide substrate.	89
4-1	Effect of *in vitro* incubation of 6% PEG 8000 extracts obtained from 1 and 7-day old rabbit hearts perfused in the presence or absence of insulin with ATP/Mg^{2+} on AMPK activity	142
4-2	Effect of 5-amino 4-imidazolecarboxamide riboside (AICAR) on AMPK activity, ACC activity and palmitate oxidation rates in 7-day old rabbit hearts.	143
5-1	Effect of AICAR and iodotubercidin and 2'deoxyadenosine on mechanical function in isolated working hearts from 7-day old rabbits.	182
5-2	Effect of AICAR and iodotubercidin on nucleotide levels in isolated working hearts from 7-day old rabbits .	183
5-3	Effect of 2'deoxyadenosine on AMPK, ACC and palmitate oxidation rates normalized for cardiac work in isolated working from 7-day old rabbits.	184
6-1	Effect of AICAR iodotubercidin and 2'deoxyadenosine on mechanical function in aerobic and reperfused-ischemic 7-day old newborn hearts.	226
6-2	Effect of ischemia and AICAR iodotubercidin and 2'deoxyadenosine on CoA ester levels at the end of perfusion in aerobic and reperfused-ischemic 7-day old newborn hearts.	227

6-3 Effect of ischemia, AICAR, iodotubercidin and 2'deoxyadenosine on nucleotide levels at the end of perfusion in aerobic and reperfused-ischemic 7-day old newborn hearts. 228

7-1 Effect of glucagon or isoproterenol on mechanical and hemodynamic functions in isolated working hearts from 7-day old rabbits. 265

7-2 Effect of glucagon or isoproterenol on nucleotide levels in 7-day old isolated perfused newborn rabbit hearts. 266

List of Figures

Figure Description Page

1-1 Proposed mechanism by which energy substrate metabolism is regulated in the heart. 29

1-2 Brief overview of fatty acid metabolism in the heart. 31

2-1 Fate of radiolabelled palmitate in the newborn heart. 66

3-1 Possible mechanism by which activation of AMPK results in a decreased ACC activity and malonyl CoA levels in the newborn rabbit heart. 91

3-2 Representative Commassie blue stained gel and immunoblot of PEG 8000 extracts from unperfused 7-day old hearts and purified rat liver AMPK. 93

3-3 Densitometric analysis of AMPK α2-subunit band intensity in PEG 8000 extracts obtained from unperfused 7-day old hearts. 95

3-4 Representative immunoblot analysis of (A) β subunit and (B) γ subunit of AMPK in PEG 8000 extracts obtained from unperfused 7-day old hearts. 97

3-5 Densitometric analysis of AMPK γ-subunit band intensity in PEG 8000 extracts obtained from unperfused 7-day old hearts. 99

3-6 Representative immunoblot analysis of (A) AMPK (B) ACC in 6% PEG 8000 extracts obtained from unperfused fetal and 1-day old rabbit hearts. 101

3-7 Effect of increasing amount of protein on AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts 103

3-8 Time dependence of AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts. 105

3-9 Effect of increasing AMP concentration on AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts 107

3-10 AMPK activity in unperfused fetal and 1-day old hearts. 109

3-11 AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts obtained in the presence or absence of sodiun fluoride and sodium pyrophosphate in the extraction buffer. 111

3-12 AMPK activity following 30 min pre-incubation of 6% PEG 8000 extracts obtained from unperfused 7-day old heart with ATP /Mg^{2+}. 113

3-13 AMPK activity following 30 min pre-incubation of 6% PEG 8000 extracts from unperfused 7-day old hearts with PP2A or PP1C. 115

3-14 ACC activity in 6 % PEG 8000 extracts from unperfused 7-day old hearts obtained in the presence or absence of sodium fluoride and sodium pyrophosphate in the extraction buffer. 117

3-15 Proposed mechanism by which increased AMPK activity may result in an increased fatty acid oxidation rates in the newborn rabbit heart. 119

4-1 AMPK activity in unperfused 1-day and 7-day old hearts. 145

4-2 Representative immunoblot analysis of AMPK in unperfused 1-day and 7-day old heart using anti-AMPK catalytic subunit polyclonal antibody. 147

4-3 Palmitate oxidation rate in 1-day and 7-day old hearts perfused in the presence or absence of insulin 100 µU/ml). 149

4-4 ACC activity in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 µU/ml). 151

4-5 AMPK activity in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 µU/ml). 153

4-6 AMPK activity in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 µU/ml) following pre-incubation with PP2A. 155

4-7 Proposed mechanism by which decreased insulin levels following birth results in an increase in fatty acid oxidation. 157

5-1 Palmitate oxidation rates in control, AICAR, and iodotubercidin perfused 7-day old rabbit hearts. 186

5-2 AMP-activated protein kinase activity in control, AICAR and iodotubercidin-treated 7-day old rabbit hearts. 188

5-3 ACC activity in control, AICAR and iodotubercidin-treated 7-day old rabbit hearts. 190

5-4 Malonyl CoA levels in control, AICAR, and iodotubercidin-treated 7-day old rabbit hearts. 192

5-5 Representative immunoblot analysis showing effect of iodotubercidin on AMPK catalytic subunit and ACC protein abundance. 194

5-6 Correlation between AMP-activated protein kinase activity and acetyl CoA carboxylase activity. 196

5-7 Correlation between AMP-activated protein kinase activity and fatty acid oxidation rates. 198

5-8 Correlation between acetyl CoA carboxylase activity and palmitate oxidation rates. 200

5-9 Proposed scheme showing mechanism by which AICAR or iodotubercidin alters fatty acid oxidation rates in the newborn rabbit heart. 202

6-1 Perfusion protocols showing different experimental groups studied using isolated working 7-day old newborn rabbit hearts. 230

6-2 AMPK activity in 6% PEG 8000 extracts from 7-day old newborn rabbit hearts obtained at the end of aerobic and at the end of ischemia. 232

6-3 ACC activity in 6 % PEG 8000 extracts from 7-day old newborn rabbit hearts obtained at the end of aerobic and at the end of ischemia. 234

6-4 Representative immunoblot analysis showing (A) AMPK and (B) ACC protein levels in 6% PEG 8000 extracts from 7-day old newborn rabbit hearts obtained at the end of aerobic and at the end of ischemia. 236

6-5 AMPK activity in 6% PEG 8000 extracts obtained from control, AICAR, iodotubercidin, or 2'deoxyadenosine-treated 7-day old newborn hearts. 238

6-6 ACC activity in 6% PEG 8000 extracts obtained from control, AICAR iodotubercidin, or 2'deoxyadenosine-treated 7-day old newborn hearts. 240

6-7 Palmitate oxidation rates in control, AICAR iodotubercidin or 2'deoxyadenosine-treated 7-day old newborn hearts. 242

6-8 Cardiac work with time during aerobic, and reperfusion following ischemia in control, AICAR iodotubercidin or 2'deoxyadenosine- treated 7-day old newborn hearts. 244

6-9 Proposed mechanism by which AMPK activation or inhibition respectively, results in a depressed or improved functional recovery in reperfused-ischemic newborn heart. 246

7-1 Palmitate oxidation rates in 7-day old isolated rabbit hearts perfused in the presence or absence of glucagon or isoproterenol. 268

7-2 AMPK activity in 7-day old rabbit hearts perfused in the presence or absence of glucagon or isoproterenol. 270

7-3 ACC activity 7-day old rabbit hearts perfused in the presence or absence of glucagon or isoproterenol. 272

7-4 Malonyl CoA levels determined in 6% perchloric acid extract obtained from 7-day rabbit hearts perfused with glucagon or isoproterenol. 274

7-5 Representative immunoblot analysis showing (A) AMPK and (B) ACC protein levels in 6% PEG 8000 extracts from 7-day old newborn rabbit hearts perfused with glucagon. 276

7-6 cAMP levels determined in 10% trichloroacetic acid extract obtained from 7-day old rabbit hearts perfused with glucagon or isoproterenol. 278

7-7 Proposed pathway by which increase in glucagon levels may contribute to the increased fatty acid oxidation rates in newborn heart. 280

8-1 Overview of proposed mechanism by which fatty acid oxidation rapidly matures in the newborn rabbit heart. 310

List of Abbreviations:

ACC:	acetyl-CoA carboxylase
ACC-265:	265 kilodalton isoenzyme of acetyl CoA carboxylase
ACC-280:	280 kilodalton isoenzyme of acetyl CoA carboxylase
AICAR:	5-amino-4-imidazolecarboxamide ribonucleoside
AMARA	AMARAASAAALARRR synthetic peptide
AMP:	adenosine monophosphate
AMPK:	5'-AMP activated protein kinase
ATP:	adenosine triphosphate
CW	cardiac work
CoA:	coenzyme A

CPT I:	carnitine palmitoyltransferase I
CPT 2:	carnitine palmitoyltransferase 2
DCA:	dichloroacetate
EGTA:	ethyleneglycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
EDTA:	ethylenediaminetetraacetic acid
HCl:	hydrochloric acid
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR:	heart rate
IgG:	immunoglobulin G
kDa:	kilodalton
mRNA:	messenger ribonucleic acid

MW:	molecular weight
NAD:	nicotinamide adenine dinucleotide
$NADH_2$:	dihydronicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis
PCA:	perchloric acid
PDC:	pyruvate dehydrogenase complex
PMSF:	phenylmethylsulfonyl fluoride
PSP:	peak systolic pressure
RNA:	ribonucleic acid
SDS:	sodium dodecylsulphate
SNFI:	sucrose nonfermenting 1

SAMS:	HMRSAMSGLHVKRR synthetic peptide
TCA:	tricarboxylic acid
TEMED	N, N, N', N'- tetramethylethylenediamine.
TOFA:	5-tetradodecyloxy-2-furancarboxylic acid

Chapter 1

Introduction

1-1 Background:

In the fetus, lactate oxidation and glycolysis are the predominant source of ATP for the developing heart (Werner and Sicard, 1987). Immediately after birth however, fatty acid oxidation rapidly increases in 7-day compared to 1-day old heart and becomes the predominant source of ATP production for the heart (Lopaschuk and Spafford, 1990). As the newborn matures, fatty acids remain the preferred oxidative substrate for high energy phosphate production in preference to carbohydrates, at least under normal aerobic conditions in adult healthy hearts (Itoi and Lopaschuk, 1993). The mechanism responsible for this rapid switch in energy substrate preference within seven days post-birth remains poorly understood.

A number of factors have been suggested to play important roles in this dramatic transition in energy substrate utilization by the newborn heart. These include changes in energy substrate availability to the heart and alterations in circulating hormonal levels (see Table 1-1). Other direct subcellular changes (involving changes in enzyme activity) within the myocardium may also be important in facilitating this rapid transition from a heart overtly reliant on carbohydrate metabolism to one which is predominantly dependent on fatty acid oxidation for energy production. The main goal of this dissertation is therefore to further our knowledge of the possible mechanism responsible for this rapid maturation of fatty acid oxidation as energy substrate for the newborn heart.

1-2 Energy substrate supply to the fetal and newborn heart:

Following conception, the heart starts to beat approximately by the third week of gestation in humans. Development of the fetal heart necessitates an increase in ATP production to support not only the energy demands of the contractile machinery, but also the growth requirements of the developing heart. Carbohydrates, especially glucose and lactate, are important energy substrates for the fetal heart (see Table 1-2). Although the circulating levels of glucose are not remarkably different in the newborn and adult animal, lactate levels differ appreciably. In the fetal circulation, lactate levels can exceed 10 mM in late gestation (Comline and Silver, 1976; Medina, 1985) (Table 1-1). These high lactate levels appear to provide the fetal heart with a substrate highly enriched in lactate. In fact, lactate oxidation can account for the majority of total oxygen consumption by the fetal heart. Interestingly, within hours of birth, plasma lactate levels decrease to 1-2 mM (Medina, 1985). The decreased plasma lactate levels in the immediate newborn period appear to decrease the contribution of lactate to total ATP production in the newborn heart.

In contrast to lactate, plasma free fatty acid levels are very low in the fetal circulation compared to levels in the newborn or adult (Table 1-1). This low level of circulating fatty acids contribute to the low fatty acid oxidation seen in fetal hearts (Table 1-2). In fact, the umbilical arterial blood concentration of free fatty acids in pregnant women was found to be approximately 0.025 mM (Phleps et al., 1981; Knopp et al., 1986). It is possible that the low fatty acid oxidation in fetal heart compared to newborn (Table 1-2) is partly due to low concentrations of fatty acids in the fetal circulation (Table 1-1).

The reason for low concentration of fatty acids in fetal circulation is not clearly understood, a transplacental free fatty acid gradient (such as is present in humans) may be a contributing factor (Knopp et al, 1986). Immediately following birth however, plasma free fatty acids increase rapidly to about 0.3 mM, a concentration that is very similar to that seen in adult circulation (Medina, 1985; Table 1-1). This rapid increase in circulating fatty acids provides newborn heart with the necessary energy substrate for ATP production. However, it is still unclear how increased availability of fatty acids result in an increased fatty acid oxidation in the newborn heart.

1-3 Maturational changes in energy substrate utilization in the newborn heart:

Glycolysis, glucose oxidation (to a lesser extent) and lactate oxidation are major sources of ATP production in 1-day old rabbit heart (Lopaschuk and Spafford, 1990). Fatty acid oxidation however becomes the predominant source of ATP in 7-day old heart (Lopaschuk et al., 1991). The reason for this increase in fatty acid oxidation is due at least in part, to increased circulating levels of fatty acid in 7-day old heart. Increased levels of fatty acids in itself may not necessarily translate into increased fatty acid oxidation. This is due primarily to the fact that fatty acids must be activated and translocated into the mitochondria for eventual oxidation by a variety of enzymes. The subcellular mechanism responsible for this decline in carbohydrate utilization and an increase in myocardial fatty acid oxidation in the immediate newborn period has yet to be clearly delineated.

1-4 Fatty acid uptake and metabolism:

Fatty acids are present in the circulation either as unesterified free fatty acids, circulating very low density lipoproteins (VLDL) or as chylomicrons (mostly as triacylglycerols). However, because of their hydrophobic nature, free fatty acids must be bound to albumin to facilitate both their solubility and transport within blood vessel lumen. To be oxidized, free fatty acids must be transported from the lumen across many hydrophilic and hydrophobic barriers to reach the inner mitochondrial membrane where they become substrate for the enzymes of the β-oxidation pathway. The mechanism by which fatty acids traverse the endothelial lining of the blood vessel and other vascular barriers to get into the cardiac myocytes is not yet fully understood. It has been suggested however, that fatty acids are taken up into cardiomyocytes by passive diffusion process or by a carrier mediated fatty acid uptake mechanism (Van der Vusse et al., 1992; Burczyynski et al., 1995). Once fatty acids are inside the cardiomyocyte, they must be transported within the aqueous cytoplasm into the mitochondria. The mechanism for this transport may involve fatty acid binding proteins (FABPs). These fatty acid binding proteins are abundant in the heart and may function primarily to transport fatty acids within the cytosol (Bass, 1988). FABPs are low molecular weight proteins (usually 14-15 kDa) capable of binding hydrophobic ligands (Van der Vusse et al., 1989; Glatz et al., 1993) and may also play a protective role in buffering intracellular long-chain fatty acyl-CoA and free radicals when their levels are elevated, especially during reperfusion following ischemia (Glatz et al., 1993; Bass, 1988).

Within the aqueous cytoplasm, fatty acids are converted (activated) to the more reactive fatty acyl CoA thioester, in a reaction which involves the hydrolysis of ATP. This conversion of fatty acids to their acyl CoA esters is usually accomplished by acyl CoA synthetase (Knudsen et al., 1993). Once activated, the fatty acyl CoA must be transported into the mitochondria. This process is facilitated by the presence of carnitine dependent enzymes (see Figure 1-1). Carnitine palmitoyltransferase 1 (CPT 1) is the rate limiting enzyme in the intra-mitochondrial uptake of activated fatty acids and catalyzes the formation of fatty acylcarnitine from fatty acyl CoA. This fatty acylcarnitine then becomes a substrate for carnitine: acylcarnitine translocase, which transfers fatty acylcarnitine into the mitochondria.

Inside the mitochondria, carnitine palmitoyltransferase 2 (CPT 2), an enzyme situated on the inner mitochondria membrane, catalyzes the release of fatty acyl CoA moiety from fatty acylcarnitine in the mitochondria matrix. This fatty acyl CoA within the mitochondria then becomes a substrate for fatty acyl CoA dehydrogenase and other enzymes of β-oxidation. The end result is the production of acetyl CoA, reduced equivalents (NADH and $FADH_2$) and a fatty acyl CoA with two carbon chain shorter than the initial starting substrate (see Schulz, 1991 and Schulz, 1994). The fatty acyl CoA with two carbon atoms shorter is then re-fed into the β-oxidation spiral until it is completely oxidized. The acetyl CoA produced is then taken up into the citric acid cycle (see Figure 1-2) with the eventual formation of $NADH_2$, $FADH_2$ and GTP. These reduced equivalents feed into the electron transport chain with subsequent oxidative phosphorylation and production of high-energy phosphates.

1-5 Regulation of fatty acid metabolism in the heart:

Malonyl CoA and CPT 1 in the heart.

Malonyl CoA is the product of a two stage enzymatic carboxylation of acetyl CoA by acetyl CoA carboxylase (ACC), a cytoplasmic enzyme (Wakil et al., 1958; Wakil et al., 1983). In both newborn and adult hearts, CPT 1 is very sensitive to inhibition by malonyl CoA (Lopaschuk et al., 1994; Kudo et al., 1995). In fact, CPT 1 can be inhibited by low nanomolar concentrations of malonyl CoA (McGarry et al., 1983). It is now clearly established that malonyl CoA produced by ACC catalytic activity is an important regulatory mechanism responsible for controlling the entry of long chain fatty acyl CoA into the mitochondria for β-oxidation (see Lopaschuk and Gamble, 1994 and Figure 1-1). The fate of malonyl CoA in the heart is, however, not yet known. Recent data in our laboratory show that malonyl CoA decarboxylase exists in newborn rabbit and adult rat hearts with measurable activity (Dyck et al., 1998a) and may be involved in the regulation of malonyl CoA levels and fatty acid oxidation in the heart. To date, the mechanism responsible for regulating malonyl CoA decarboxylase activity in newborn heart remains poorly understood.

An important substrate for CPT 1 in the heart is L-carnitine. In newborn circulation, L-carnitine levels are quite low. Circulating L-carnitine levels, however, increase as the animal matures (Brown et al, 1995). It is conceivable that increased L-carnitine levels may facilitate increased fatty acid uptake and oxidation in newborn heart since carnitine is a key substrate for CPT 1, the rate-limiting enzyme in intra-mitochondria uptake of fatty acids.

CPT 1 is an important target for the regulation of fatty acid oxidation and has been proposed to play important role in the maturation of fatty acid oxidation in newborn heart. Recent studies by Brown et al. (1995) demonstrate a CPT 1 isoform switch in the newborn period in rat hearts. Within the first few days of life in the rat heart, an 82 kDa CPT 1 isoform with a low Km for carnitine predominates, and accounts for almost 70% of total CPT 1 activity, while an 88 kDa CPT 1 isoform accounts for the rest of CPT 1 enzymatic activity. As the animal matures, however, an isozyme shift occurs resulting in the predominance of an 88 kDa CPT 1 isoform with a higher Km for carnitine. This 88 kDa isoform is less sensitive to inhibition by malonyl CoA. It would appear that the flux of activated fatty acyl CoA through the CPT 1 is not only dependent on the switching of CPT 1 isoform expressed in the newborn period, but also on the cytoplasmic concentrations of both malonyl CoA and L-carnitine.

Studies by Xia et al. (1996) also demonstrate that CPT 1 isoform expression can change in cultured cardiac myocytes. Using cultured neonatal rat myocytes, these investigators showed that electrical stimulation of cardiomyocytes resulted in an increased mRNA for 82 kDa CPT 1 isoform. Whereas CPT 1 isoform expression changes in the newborn heart, the significance of this change to the maturation of myocardial fatty acid oxidation in the immediate newborn period is not completely understood. For instance, increased expression of 82 kDa isoform of the CPT 1 should make the mitochondrial uptake of fatty acids more sensitive to inhibition by malonyl CoA. This scenario in itself would not be expected to occur in a maturing heart in which fatty acid uptake by the mitochondria increases. Further clarification into the role of changes in CPT 1 isoform in the newborn heart, especially as it relates to increased fatty acid oxidation is necessary.

Whereas the role of CPT 1 isoform switch and L-carnitine levels in the regulation of fatty acid oxidation remains poorly understood, studies from our laboratory have demonstrated that a dramatic decrease in levels of malonyl CoA occur in 7-day compared to 1-day old rabbit heart. This decrease in levels of malonyl CoA is important in the increased myocardial fatty acid oxidation seen in 7-day old heart (Lopaschuk et al, 1994). The decrease in levels of malonyl CoA, however, directly results from a decrease in ACC activity and is accompanied by an increase in fatty acid oxidation (Lopaschuk et al., 1994). It is likely, that the rapid maturation of fatty acid oxidation in newborn heart results from changes in sub-cellular mechanism responsible for controlling ACC activity in the newborn heart.

1-6 ACC and its role in fatty acid oxidation in the newborn heart

Acetyl CoA carboxylase is the rate-limiting enzyme which catalyzes the first committed step in fatty acid biosynthesis in the liver by two stage carboxylation of acetyl CoA to malonyl CoA (Wakil et al., 1958; Wakil et al., 1983). ACC is present in the heart (Thampy, 1989) and regulates fatty acid oxidation via its production of malonyl CoA (Saddik et al., 1993; Lopaschuk and Gamble, 1994). ACC is a biotin-containing enzyme which can be resolved into a catalytic biotin-carboxylase domain and a carboxyl transferase domain as deduced from cloning and amino acid analysis of chicken and rat liver ACC (Takai et al., 1988; Hardie, 1989). Two isoforms of ACC have been reported in rat heart, a 280 kDa and a 265 kDa isozymes (Thampy, 1989). Interestingly, the newborn rabbit heart also expresses both isoforms of ACC with ACC 280 kDa being the predominant ACC isoform (Lopaschuk et al., 1994). Accumulating evidence from both rat and rabbit studies now convincingly demonstrate that both isoforms of ACC can be phosphorylated with resultant decrease in ACC activity (Dyck et al., 1998b).

In rat liver, ACC 265 can be regulated over two different time frames. A rapid, short term regulation occurring in minutes and a long term (days or weeks) regulation involving distinct ACC gene promoter regions and altered rates of transcription, synthesis and degradation of ACC (Lopez-Casillas and Kim, 1989; Lopez-Casillas et al., 1991). ACC activity can be stimulated by citrate, an allosteric activator of ACC activity in the liver and an immediate precursor of cytoplasmic acetyl CoA (citrate is converted to acetyl CoA by the action of ATP-citrate lyase). For this reason, citrate is also regarded as a "feed forward activator" of ACC (Thampy and Wakil, 1985; Thampy and Wakil, 1988). It is yet unclear whether citrate allosterically activates cardiac ACC especially in newborn rabbit hearts.

Studies by Lopaschuk et al. (1994) demonstrate that ACC activity is higher in 1-day old rabbit heart, compared to 7-day old heart, and is responsible for the higher levels of malonyl CoA in 1-day compared to 7-day old heart. High levels of malonyl CoA in 1-day old heart therefore appear to be primarily responsible for the decreased fatty acid oxidation compared to 7-day old heart, secondary to an inhibition of CPT 1 activity (Lopaschuk et al., 1994). This observation supports the notion that a decrease in malonyl CoA production by decrease in ACC activity is an important mechanism responsible for increased fatty acid oxidation following birth. Unfortunately, the reason for decreased ACC activity in the newborn heart remains unknown as at the beginning of my studies.

Recent studies in rat liver demonstrate that ACC can be phosphorylated and inactivated by a novel AMP-activated protein kinase (Carling et al., 1989; Hardie, 1992). A possible candidate kinase responsible for inactivating ACC in the newborn heart therefore appears to be AMPK. Whether or not AMPK actually exists in the newborn heart remained to be investigated as at the beginning of my studies

1-7 AMPK and its involvement in the regulation of ACC in the heart

AMPK is a multisubstrate protein kinase which consists of α catalytic subunit (63 kDa) and β (40 kDa) and γ (38 kDa) regulatory subunit. (Gao et al., 1995; Gao et al., 1996; Stapleton et al., 1997). At present, the function of the non-catalytic β and γ subunits are not immediately clear, but recent studies showed that all three subunits are required for both the formation of a stable heterotrimeric complex as well as an increased catalytic activity of AMPK (Dyck et al, 1996). All subunits (α, β, γ) of AMPK have recently been cloned and found to exist as two or more distinct gene products. For instance, the catalytic subunit exists as two isoforms termed α1 and α2. In addition, the β subunit exists as β1 and β2. The γ subunit however exists as γ1, γ2, γ3 isoforms respectively (Woods et al, 1996; Stapleton et al., 1997). Recent evidence now demonstrate that α2 isoform of AMPK is preferentially localized to the nucleus (Salt et al., 1998). The function of each of the different isozymes of both catalytic and regulatory subunits of AMPK has yet to be fully elucidated.

The α1 isoform of AMPK is widely expressed in mammalian tissues, whereas α2 isoform is highly expressed in oxidative tissues such as the heart and skeletal muscle (Stapleton et al, 1997). Interestingly, both α catalytic and regulatory β γ subunits of AMPK have been shown to have yeast homologues. For instance, *Saccharomyces cerevisae* expresses the *SNF 1, SIP 1/SIP 2* and *SNF 4* genes which, respectively, have high degree of sequence identity to α, β, and γ subunits of AMPK (Gao et al., 1995; Mitchelhill et al., 1994). The high degree of sequence identity between yeast and mammalian homologues provides genetic evidence that AMPK may have been highly conserved throughout evolution (Hardie and MacKintosh, 1992).

Although the role of AMPK in the mammalian system is not yet fully understood, accumulating evidence suggest that AMPK may be a "fuel gauge" for the mammalian cell. For instance, in the liver, AMPK may serve to turn off energy consuming biosynthetic pathway such as fatty acid biosynthesis and cholesterol synthesis and switch on alternative pathways for ATP generation such as fatty acid oxidation during conditions of cellular stress. It is possible that in the heart, AMPK also serves as a "fuel gauge" during conditions of cellular stress. This cellular stress may include heat shock, arsenite or fructose treatment, and prolonged exercise or ischemia (Hardie, 1992; Witters et al., 1991; Gillespie and Hardie, 1992; Kudo et al., 1995).

These conditions of cellular stress increase AMP levels and AMP/ATP ratio, resulting in an activation of AMPK. It has now been demonstrated that AMPK can be activated in either of four ways by elevated AMP levels. First, by a direct allosteric activation involving the binding of AMP to a yet to be fully characterized AMP site on AMPK. Second, AMP can indirectly activate an upstream AMPK kinase (AMPKK) resulting in an increased phosphorylation and activation of the downstream AMPK. Third, AMP can facilitate the phosphorylation of AMPK by the upstream AMPKK (Weekes et al, 1994; Hawley et al., 1995; Moore et al., 1991). This AMPKK has recently been characterized in the rat liver (Hawley et al, 1996). Fourth, increased 5'AMP levels not only facilitate phosphorylation of AMPK by AMPKK, it also results in an inhibition of dephosphorylation of AMPK by protein phosphatases such as protein phosphatase 2A (PP2A) (Davies et al, 1995). This inhibition of PP2A prevents dephosphorylation and inactivation of AMPK. This ensures that AMPK is in a state of maximal catalytic activity (Davies et al., 1995). Accumulating evidence now demonstrate convincingly that AMPK can phosphorylate and inactivate ACC not only in rat liver but also in adipocytes (Sullivan et al., 1994), skeletal muscle (Winder et al., 1997) and heart muscle (Kudo et al., 1995; Kudo et al., 1996; Dyck et al., 1998b).

AMPK has been recently characterized in the rat heart (Kudo et al., 1996), and it plays a role in the increased fatty acid oxidation in reperfused ischemic heart via its phosphorylation and inactivation of ACC. In their study, (Kudo et al, 1996) a transient period of global no flow ischemia was sufficient to activate AMPK. Moreover, increased AMPK activity was associated with a decrease in ACC activity and an increase in fatty acid oxidation in the reperfused ischemic heart. At the onset of my studies, it was unknown whether or not AMPK actually existed in the newborn heart. In addition, it was not known if AMPK phosphorylated and inactivated ACC in newborn heart with resultant decrease in

ACC activity and an increase in fatty acid oxidation. Studies presented in this thesis therefore attempt to further elucidate the involvement of AMPK in the maturation of fatty acid oxidation in newborn heart.

1-8 Possible mechanism for the maturational changes in fatty acid oxidation in newborn heart:

The mechanism responsible for the rapid increase in myocardial fatty acid oxidation in the early newborn period remains poorly understood. A number of theories have been put forward by different investigators in an attempt to have a better understanding of the molecular mechanism(s) responsible for this dramatic change in energy substrate utilization by the heart in the immediate newborn period. Earlier studies in the liver showed that a decrease in the sensitivity of CPT 1 to malonyl CoA's inhibition could explain the increased fatty acid oxidation in rat liver in early newborn period (Prip-Buus et al., 1990). In the heart however, the sensitivity of CPT 1 to malonyl CoA's inhibition is not significantly altered (Lopaschuk et al., 1994; Kudo et al., 1995). These observations suggest that mechanisms other than changes in CPT 1 sensitivity to malonyl CoA must be responsible for the increased fatty acid oxidation in newborn rabbit heart.

A newly emerging theory that remains to be investigated in newborn rabbit heart is the possibility that increased circulating fatty acids directly induce the CPT 1 gene expression and enzyme activity. Since CPT 1 is the rate-limiting enzyme for mitochondria fatty acid uptake, changes in CPT 1 gene expression may profoundly alter fatty acid metabolism. In pancreatic β-cell line (INS-1), prolonged exposure of the cells to high concentration of palmitate rapidly increase CPT 1 mRNA by 4-6 fold. This increased CPT 1 mRNA is accompanied by a 2-3 fold increase in fatty acid oxidation in cultured cells incubated with fatty acids when compared to control incubation (Assimacopoulos-Jeannet et al., 1997). Since circulating fatty acid levels increase in the immediate newborn period, it is conceivable that increased fatty acid concentrations may result in an increased CPT 1 enzyme activity secondary to an increase in CPT 1 mRNA. This possibility therefore merits further investigation.

Another theory in an attempt to explain the mechanism responsible for the maturational changes in fatty acid oxidation has been put forward by Brown et al. (1995). These investigators suggest that a shift in CPT 1 isoform from an 82 kDa to an 88 kDa CPT 1 isoform in the immediate newborn period could be a possible explanation for the increased fatty acid oxidation in the developing rat heart. Plausible as this explanation appears, it is not a satisfactory explanation for increased maturation of fatty acid oxidation in the newborn heart. This is due to the fact that malonyl CoA is a potent inhibitor of CPT 1 activity in isolated rat hepatocytes, liver homogenates and the rat heart (McGarry et al., 1983; Thampy, 1989; Awan and Saggerson, 1993; King et al., 1988 Lopaschuk et al., 1995). Regardless of CPT 1 isozyme expressed therefore, malonyl CoA levels within the heart remain an important determinant of both CPT 1 activity and uptake of fatty acids into the mitochondria due to its inhibitory effects on CPT 1. As stated earlier, malonyl CoA is produced by ACC, an important regulator of myocardial fatty acid oxidation (Saddik et al., 1993; Lopaschuk and Gamble, 1994). In understanding the mechanism responsible for increased fatty acid oxidation in newborn heart, adequate understanding of the molecular mechanism responsible for changes in ACC activity and malonyl CoA levels is necessary.

Based on available evidence from our laboratory and others (Kudo et al., 1995; Kudo et al., 1996; Winder et al., 1997; Merrill et al., 1997), we propose that increase in AMPK activity regulates myocardial fatty acid oxidation secondary to a decrease in ACC activity. This proposal is supported by recent studies in the heart which showed that increased fatty acid oxidation in the reperfused ischemic heart is associated with an increase in AMPK activity and accompanied by a decrease in ACC activity (Kudo et al., 1995). Pharmacological agents like AICAR have been demonstrated to activate AMPK, decreased ACC activity and increased overall fatty acid oxidation in rat skeletal muscle (Merrill et al., 1997). It is possible that AMPK, ACC and fatty acid oxidation pathway may be regulated in a similar fashion in newborn rabbit heart as in rat heart and skeletal muscle. The postulated increase in AMPK activity resulting in decreased ACC activity, however, is consistent with the mature heart being in a position to effectively utilize fatty acids as energy substrate.

As eluded to earlier, a dramatic increase in fatty acid oxidation occurs in 7-day compared to 1-day old heart. However, changes in circulating levels of insulin and glucagon occur within the same period. The effect of insulin or glucagon on AMPK, ACC and increased myocardial fatty acid oxidation in the newborn heart therefore merits further investigation.

1-9 Hormonal changes in newborn heart and its effects on fatty acid metabolism:

Circulating blood insulin levels decrease rapidly immediately following birth, this decrease is accompanied by an increase in circulating glucagon levels (Girard et al., 1992). Although the reason for these changes in hormonal levels have not been fully elucidated, previous studies from our laboratory have shown that insulin plays a role in the rapid increase in myocardial fatty acid oxidation in the immediate post-natal period (Lopaschuk et al., 1994). Insulin increases ACC activity and decreases AMPK activity in isolated rat hepatocytes (Witters and Kemp, 1992). Interestingly, glucagon can significantly decrease ACC activity in isolated rat liver secondary to stimulation of AMPK (Sim and Hardie, 1988).

Assuming that the newborn heart expresses an active AMPK, we propose that the decreased circulating insulin levels following birth results in an increased AMPK activity and a concomitant increased phosphorylation and inactivation of ACC. Since glucagon decreases ACC activity in hepatocytes via stimulation of AMPK (Sim and Hardie, 1988) it is conceivable that increase in circulating glucagon levels may result in an activation of AMPK with resultant decrease in ACC activity. Both the rapid decline in insulin levels and the dramatic increase in glucagon levels in the immediate newborn period may act together to decrease ACC activity in the immediate newborn period. The result of this would be a decrease in malonyl CoA levels, thereby relieving the inhibitory effects of malonyl CoA on CPT 1. This would result in an increased uptake and oxidation of fatty acids within the mitochondria. This possibility was addressed in my studies.

1-10 Modulation of energy substrate metabolism as a therapeutic target:

Studies described in this thesis attempt to further our understanding of the sub-cellular mechanism responsible for the maturation of fatty acid oxidation in the newborn heart. It is possible that knowledge gained from my studies would improve in general our understanding of how fatty acids are metabolized within the cardiac myocyte. Such knowledge may be manipulated and exploited to therapeutic advantage.

An over-reliance of the heart on the oxidation of fatty acids as a source of energy substrate for ATP production is detrimental to cardiac function (Lopaschuk et al., 1994b). For instance, in diabetic rats, excessive reliance of the heart on utilization of fatty acids as energy substrates has been associated with poor functional recovery of cardiac function following ischemia (see Lopaschuk et al., 1993). This excessive oxidation of fatty acids in the reperfused-ischemic heart results in both a depressed glucose utilization and poor cardiac performance in the post-ischemic period by a yet to be fully understood mechanism. The detrimental effect of high fatty acids oxidation on cardiac function may be attributed at least in part to the inhibitory effects of high levels of fatty acid oxidation on glucose oxidation (Lopaschuk et al., 1994b). Decreased glucose oxidation would further uncouple glycolytic rates from glucose oxidation rates resulting in proton production from hydrolysis of glycolytically derived ATP. This may lead to acidosis, calcium overload and depressed cardiac function during reperfusion period. It is conceivable therefore that compounds which increase glucose oxidation and/or decrease fatty acid oxidation may result in an improved cardiac performance.

Indeed, dichloroacetate (DCA), an agent which increases glucose oxidation rates has been shown to improve the recovery of the heart function in the reperfused-ischemic rat heart (McVeigh and Lopaschuk, 1990). DCA stimulates pyruvate dehydrogenase complex (PDC) by directly inhibiting the pyruvate dehydrogenase (PDH) kinase and thus inhibiting phosphorylation and inactivation of PDC (see McCormack et al., 1990 for review). In addition, ranolazine, a piperazine compound can improve heart function in the reperfused-ischemic heart. Although the mechanism of action of this compound is not yet fully elucidated, ranolazine has been shown in our laboratory to specifically inhibit β-oxidation of fatty acids (McCormack et al., 1996). The indirect result of inhibition of fatty acid oxidation is an increase in acetyl CoA production from glucose oxidation.

Carnitine and L-propionyl carnitine are another group of compounds that stimulate glucose oxidation rates and improve mechanical function during reperfusion of previously-ischemic hearts (Broderick et al., 1993). Carnitine and L-carnitine also improved cardiac performance in the hypertrophied myocardium (Schonekess et al., 1995). The increased glucose oxidation rates observed with L-carnitine and related L-carnitine derivatives occur secondary to the ability of these group of compounds to remove acetyl CoA from the mitochondria and into the cytosol, thereby lowering mitochondria acetyl CoA / CoA ratio. The end result is a stimulation of glucose oxidation secondary to an activation of pyruvate dehydrogenase complex.

Another group of agents with effect on energy metabolism are the inhibitors of CPT 1, including etomoxir and oxfenicine. Improved functional recovery in the reperfused-ischemic hearts following etomoxir treatment have also been associated with the indirect increase in glucose oxidation rates arising from the decreased fatty acid oxidation (Lopaschuk et al., 1989). Unfortunately, etomoxir produces cardiac hypertrophy in the clinical setting by a yet to be fully understood mechanism. Thus the search for newer compounds which decreases fatty acid oxidation or increases glucose oxidation preferentially to improve cardiac performance or efficiency continues to occupy a key research focus.

Other agents which inhibit ACC activity [e.g. 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA)] have been explored with limited success (Dr. James Gamble personal communications). It is possible that compounds, which increase ACC activity by inhibiting AMPK activity, may decrease fatty acid oxidation in the heart. It is also possible that such agents may improve heart function in the reperfused-ischemic newborn (and possibly adult) hearts secondary to an increased ACC activity and a decreased fatty acid oxidation.

Myocardial ischemia is a real phenomenon in the newborn because some newborn will need to undergo surgical procedure to correct congenital heart defect such as transposition of great valves, the patent ductus arteriosus or atrial septal defect. In some cases, heart function following surgery remains poor. In a recent study, elevated levels of fatty acid were observed in infants and adults during and following surgery (Lopaschuk et al., 1994). These high levels of fatty acid contribute significantly to the poor functional recovery of the heart following surgery. Inhibition of fatty acid oxidation in the heart by AMPK inhibitors may prove to be novel therapeutic approach to improve the recovery of the heart following surgery.

1-11 The purpose of this study:

The main purpose of this research is to investigate the possible involvement of AMPK in the maturation of fatty acid oxidation in the newborn heart. To achieve this purpose, I set out to investigate whether or not AMPK is involved in the rapid increase in fatty acid oxidation in newborn heart. Furthermore, since accumulating evidence (Lopaschuk et al., 1992; Kudo et al., 1995) suggest that excessive rates of fatty acid oxidation are detrimental to functional recovery of reperfused-ischemic heart, I determined if inhibition of AMPK activity is associated with decreased fatty acid oxidation and improved functional recovery in the reperfused-ischemic hearts. The rationale for carrying out these studies is based on the fact that some neonates have to undergo corrective surgery to repair a congenital heart defect, which often involve temporary cessation of heart function as the patient is placed on a heart-lung machine during surgical procedure. The need therefore arises to efficiently optimize energy substrate utilization by the heart with a view to adequately protect the newborn heart before, during and following surgery.

1-12 The central hypothesis in this thesis:

I hypothesize that AMPK regulates myocardial fatty acid oxidation in newborn heart secondary to alteration in ACC activity.

1-13 Aims and objectives of this thesis:

In testing above stated hypothesis, my aim was to demonstrate that AMPK is involved in the regulation of myocardial fatty acid oxidation rates in the newborn heart. To achieve this aim, my key objectives were to:

1) demonstrate that newborn heart expresses an active AMPK which is involved in regulating ACC
2) determine the relationships between AMPK, ACC and fatty acid oxidation in newborn heart
3) investigate the involvement of AMPK in the rapid maturation of fatty acid oxidation in newborn heart
4) evaluate the effect of AMPK modulators on fatty acid oxidation in the newborn heart under aerobic conditions
5) determine the effect of AMPK modulators on fatty acid oxidation and functional recovery following ischemia in newborn heart and
6) determine the effect of glucagon on AMPK activity and fatty acid oxidation in newborn heart.

If AMPK regulates fatty acid oxidation in newborn heart, AMPK inhibitors may find therapeutic use in protecting the heart from reperfusion injury during and following ischemic episode.

1-14 Methodological considerations:

In carrying out these studies outlined in this thesis, fetal, 1-day and 7-day old rabbit hearts were used as experimental model. This is partly because newborn rabbit hearts have been extensively studied in our laboratory and earlier findings of the increase in fatty acid oxidation which was associated with decreased ACC activity were first observed in this experimental model (Lopaschuk and Spafford, 1990; Lopaschuk et al., 1991; Lopaschuk et al., 1994; Itoi and Lopaschuk, 1996). Moreover, newborn rabbits are relatively easy to obtain and are relatively non-aggressive. To carry out these studies, in some instances, newborn rabbit hearts were unperfused (i.e. hearts were quickly excised from the animal and briefly rinsed in Krebs'-Henseleit solution and immediately frozen in liquid nitrogen). This allows the assessment of enzyme activity prior to perfusion. In other instances, hearts were perfused in either Langendorff mode or in the working mode, as described in Methods. This allowed me to measure energy metabolism under conditions whereby contractile work performed by the heart could also be monitored. The procedure also has additional advantage of allowing me to determine the activity of key enzymes involved in energy metabolism at the end of perfusion protocol as described in Methods.

Energy metabolism has also been studied in cardiac myocytes (Abdel-aleem et al., 1996; Xia et al., 1996). This method employs a collagenase digestion of the heart cells. However, the main disadvantage of this method is that it is not possible to put a workload on these myocytes to simulate a condition very similar to what happens in the intact organ. McMillin's group (Xia et al., 1996) have used cultured neonatal myocytes to study changes in CPT 1 expression in response to electrical stimulation. Studies using cardiac myocytes are thus very useful in studying the involvement of various enzymes in regulation of energy metabolism. Unfortunately, cardiac myocytes under this condition are quiescent and exhibit very low metabolic demand.

Another possible approach for measuring metabolism is to measure differences in substrate concentration in both arteries and veins, and then use the differences in substrate concentration across arterial and venous circulation as an index of substrate metabolism. Bing et al. (1953) previously employed this methodology to study carbohydrate metabolism in the human heart. In addition, this method was previously employed by Fisher et al. (1980) to study myocardial oxygen, glucose, lactate and pyruvate consumption in chronically instrumented fetal and adult sheep. This method therefore appears to be suited for studies involving large experimental animals e.g. pigs, lambs, and sheep. Another possible drawback of this approach is that not all substrate extracted from the blood may be completely oxidized.

1-15 Expected results from studies in this thesis:

The mechanism responsible for the increased fatty acid oxidation in 7-day compared to 1-day old heart remains poorly understood. However, ACC activity has been demonstrated to decrease in 7-day compared to 1-day old heart. At the beginning of my studies, the mechanism responsible for the decrease in ACC activity in 7-day old heart remained unknown. Since AMPK can phosphorylate and inactivate ACC in rat liver, I expected that results of experiments described in this thesis would demonstrate that AMPK abundance and/or activity increases in 7-day compared to 1-day old heart, resulting in decreased ACC activity in 7-day old heart. It was further my expectation that AMPK inhibitors would decrease fatty acid oxidation secondary to an increase in both ACC activity and levels of malonyl CoA. Moreover, since high rates of fatty acid oxidation are detrimental to functional recovery in reperfused-ischemic hearts, I also expected that AMPK inhibitors would improve functional recovery in the newborn heart secondary to a decrease in rate of fatty acid oxidation. Overall, it was my expectation that overwhelming evidence would be accumulated to demonstrate that AMPK is not only present in newborn hearts but also is involved in the maturation and regulation of fatty acid oxidation, secondary to changes in ACC activity.

Table 1-1:

Overview of changes in circulating substrate and hormone levels in the fetus, 1 week-old and adult experimental animals.

Parameter	Fetus	1 Week-old	Adult
Substrate Levels:*			
Glucose (mM)	2.3	6	5.0
Lactate (mM)	10	2	0.5
Fatty acids (mM)	0.02	0.3	0.4
Hormonal levels:†			
Insulin (μU/ml)	95	10	4
Glucagon (ng/ml)	0.18	1	0.11
Triiodothyronine (ng/dl)	7	18	90
Corticosterone (μg/dl)	N.D	1	5

* Values for substrate levels are adapted from Medina, (1985).

† Values of hormonal levels are adapted from Girard et al. (1992).

Table 1-2:

Overview of normal contribution of energy substrates to total ATP production in fetal, 1-day old, 1 week-old and adult hearts.

Parameter	Fetal†	1-Day†	1 Week-old†	Adult†
		(% contribution to total ATP)*		
Substrate:				
Lactate	40-60	10	5	5
Glucose	10	40	25	10
Palmitate	≤ 10	≤ 20	60	70

* Variable glycolytic rates may account for the remainder of ATP production.

† Values are adapted from Werner and Sicard, (1987) from studies in fetal pig hearts.

† Values are adapted from Lopaschuk and Spafford, (1990); Lopaschuk et al. (1991); Itoi and Lopaschuk, (1993) from studies in newborn rabbit hearts.

Figure 1-1:

Proposed mechanism by which energy substrate metabolism is regulated in the heart.

ACC is an important regulator of myocardial fatty acid oxidation via its production of malonyl CoA, a potent inhibitor of the carnitine palmitoyltransferase 1 (CPT 1, the rate-limiting step in intra-mitochondria transfer of activated fatty acids). We propose that decreased ACC activity would decrease malonyl CoA levels. Decrease in levels of malonyl CoA then results in an increased activity of CPT 1 enzyme system. The uptake and β-oxidation of fatty acids would increase under this condition.

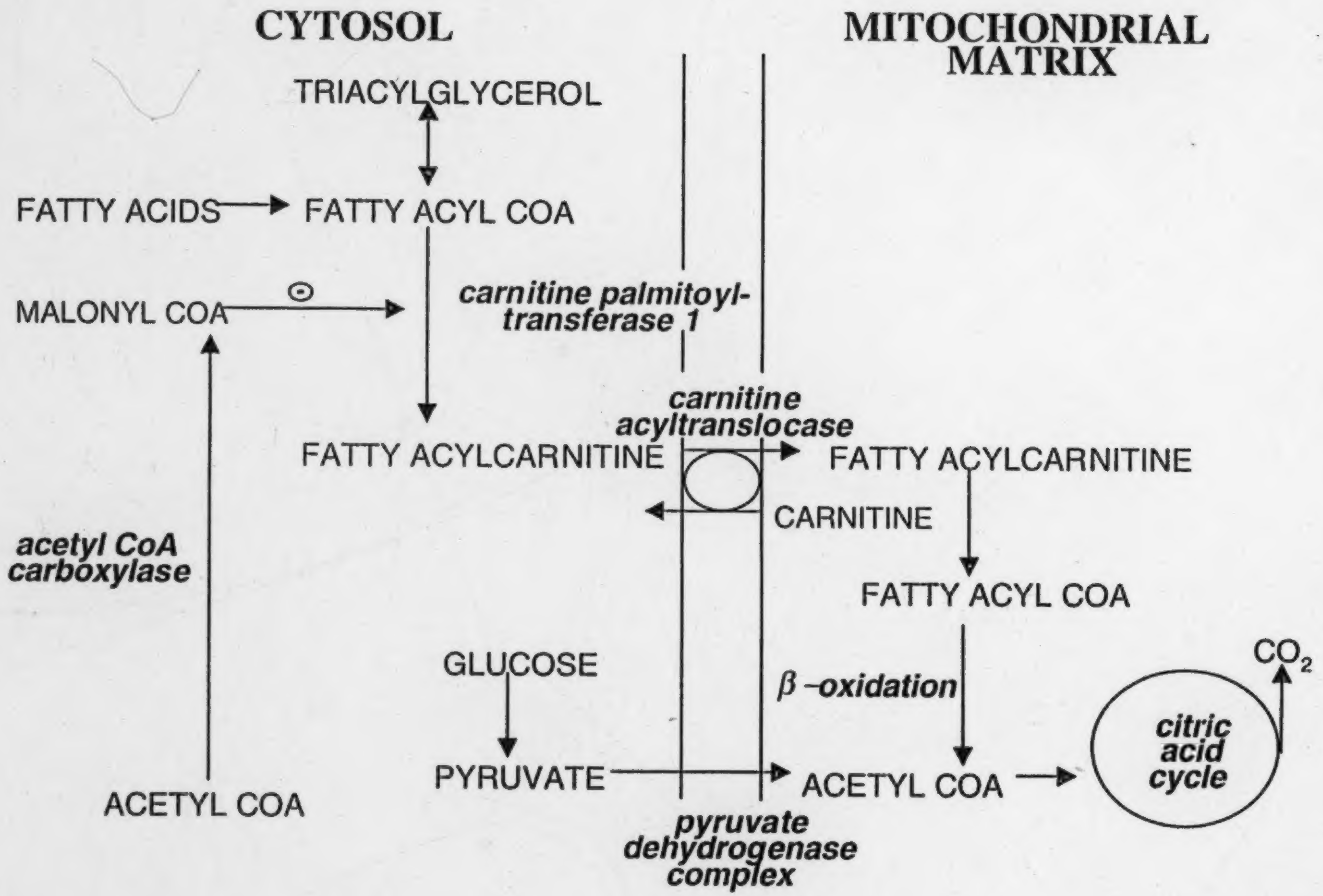
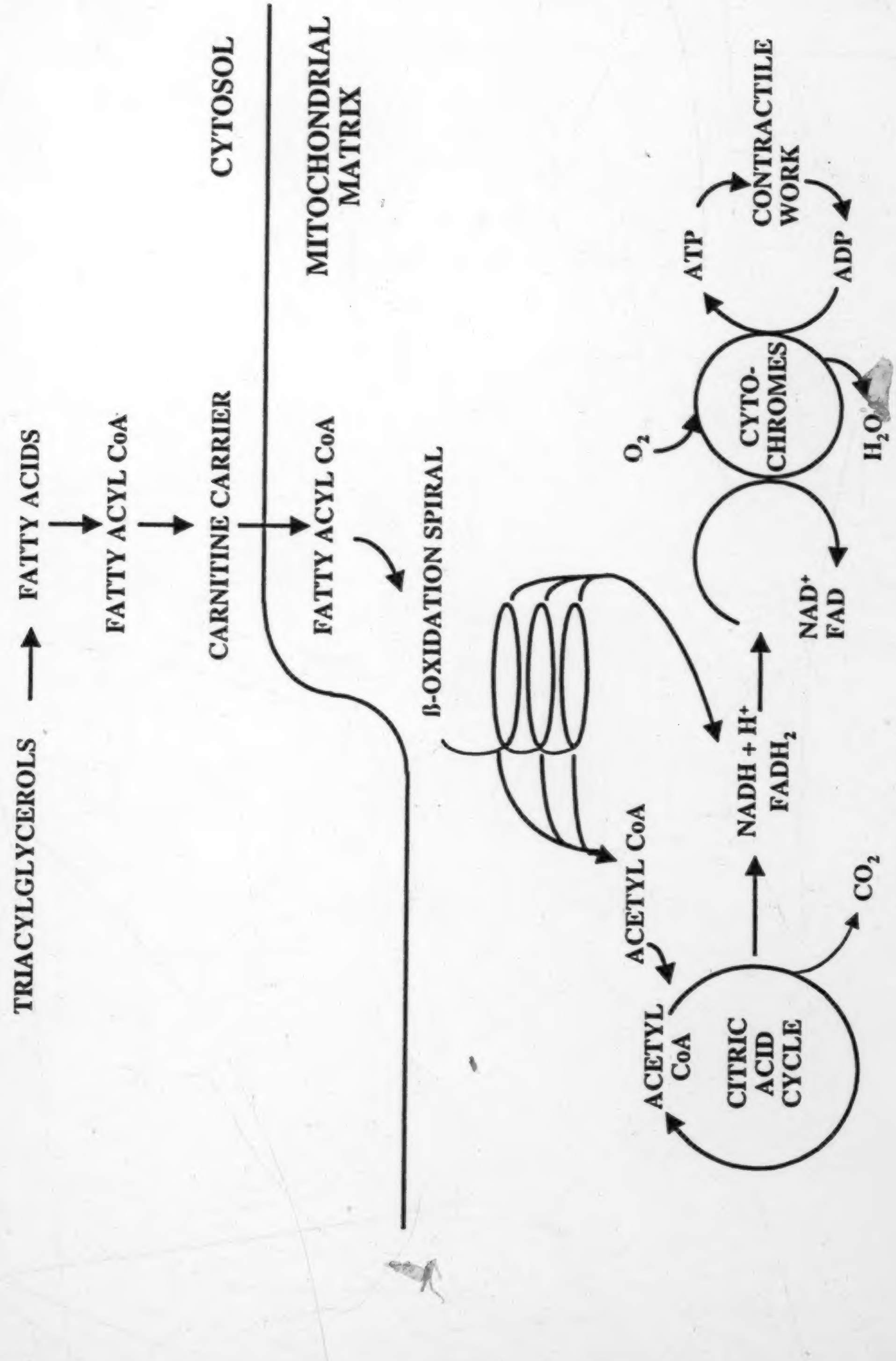
CYTOSOL
MITOCHONDRIAL MATRIX
TRIACYLGLYCEROL
FATTY ACIDS
FATTY ACYL COA
MALONYL COA
⊙
carnitine palmitoyl-transferase 1
carnitine acyltranslocase
FATTY ACYLCARNITINE
FATTY ACYLCARNITINE
CARNITINE
acetyl CoA carboxylase
FATTY ACYL COA
GLUCOSE
β -oxidation
CO₂
citric acid cycle
PYRUVATE
ACETYL COA
ACETYL COA
pyruvate dehydrogenase complex

Figure 1-2:

Brief overview of fatty acid metabolism in the heart.

Fatty acids in the cytosol are activated to fatty acyl CoA by acyl CoA synthetase. Thereafter, carnitine palmitoyltransferase 1 (CPT 1) catalyzes the transfer of carnitine to the acyl CoA moiety with the formation of fatty acylcarnitine. This fatty acylcarnitine enters the mitochondria where it is acted upon by carnitine palmitoyltransferase 2 (CPT 2) with the resultant release of fatty acyl CoA. The fatty acyl CoA then feeds into the β-oxidation spiral with the subsequent production of acetyl CoA for citric acid cycle.


TRIACYLGLYCEROLS
FATTY ACIDS
FATTY ACYL CoA
CARNITINE CARRIER
CYTOSOL
MITOCHONDRIAL MATRIX
FATTY ACYL CoA
β-OXIDATION SPIRAL
ACETYL CoA
NADH + H⁺
FADH₂
NAD⁺
FAD
ACETYL CoA
ACETYL CoA
CITRIC ACID CYCLE
CO₂
O₂
CYTO-CHROMES
H₂O
ATP
ADP
CONTRACTILE WORK

References:

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Chapter 2

General Experimental Methods and Materials

2. General Experimental Methods:

2-1 *Experimental animals used in the study:*

Fetal (29-day gestation), 1-day and 7-day old New Zealand White rabbits of either sex were used in these studies. Animals were cared for according to the guidelines outlined by the Canadian Council for Animal Care.

Preparation of fetal rabbit hearts:

On the day of experimentation, the pregnant doe (29-day gestation) was anaesthetized with sodium pentobarbital (Euthanyl® 60 mg/kg) via the lateral ear vein. Following anesthesia, fetal rabbits were removed from the doe's uterus and each rabbit was immediately injected with sodium pentobarbital (30 mg/kg, i.p). Following a two min period, the thoracic cage was opened and the hearts were quickly excised, rinsed briefly in ice-cold Krebs'-Henseleit solution (pH 7.4) and immediately frozen using Wollenberger tongs pre-cooled to the temperature of liquid nitrogen. All hearts were stored at –80 ^{0}C until ready for analysis.

Preparation of newborn rabbit hearts:

1-day and 7-day old rabbits were separated from the mother on the day of experimentation. Both 1-day and 7-day old rabbits were then immediately injected with sodium pentobarbital (60 mg/kg, i.p). Once the animals lacked sensation, a thoracotomy was performed and the exposed hearts (alongside with the lungs) were quickly excised and placed in ice-cold Krebs'-Henseleit solution. Following a brief rinsing in Krebs'-Henseleit solution, hearts were either quickly frozen in liquid nitrogen (unperfused) and stored away for subsequent tissue analysis or the aorta was cannulated for isolated heart perfusions (see below).

2-2 *Heart Perfusions:*

Initial Cannulations:

Hearts removed from newborn rabbits were initially perfused in the Langendorff mode, as previously described (Lopaschuk et al., 1994). Briefly, following cannulation of the aorta, Krebs'-Henseleit solution was delivered to the heart via the aorta at 60 mm Hg pressure. All hearts were perfused at 37 ^{0}C with oxygenated (95%O_2 /5%CO_2) Krebs'-Henseleit solution (pH 7.4) containing 11 mM glucose, 0.4 mM [1-^{14}C]palmitate bound to 3% bovine serum albumin, 3.0 mM calcium chloride and 0.5 mM EDTA. Hearts were perfused for a 40 min aerobic perfusion period during which palmitate oxidation rates were determined (as described in appropriate sections below) In specific protocols, hormones or drugs were added to the Krebs'-Henseleit solution as outlined in Methods section of individual studies. At the end of the perfusion protocol, hearts were quickly frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen. The atria was dried overnight in an oven at 100 ^{0}C and then weighed. This weight was used to calculate the total dry weight of the heart.

Isolated working heart perfusions:

Once hearts stabilized on the perfusion apparatus as described above, they were switched to perfusion in the working mode (see Barr and Lopaschuk, 1997) by initiating the flow of perfusate through the left atria and subjecting the newborn hearts to a preload pressure of 7.5 mm Hg and an afterload pressure of 30 mm Hg (as previously described by Itoi and Lopaschuk, 1996). The isolated working hearts were perfused with Krebs'-Henseleit solution containing various hormones or drug additions as specified in the Methods sections of individual studies.

All studies using isolated working hearts employed spontaneously beating hearts. Heart rate and peak systolic pressure were measured by a Gould P21 pressure transducer installed in the aortic outflow line and visualized using a Grass Polygraph model 5D. Cardiac output, aortic outflow and coronary flow were measured using in-line transonic flow probes installed in both the atrial inflow and aortic outflow lines. The flow readings were acquired by connecting the flow probes to a T 101 transonic flow meter. Cardiac work was determined as the product of the peak systolic pressure x cardiac output. At the end of the perfusion protocol, ventricular tissues were quickly frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen and stored at –80 °C until ready for analysis.

2-3 Determination of Palmitate Oxidation Rates:

Steady state palmitate oxidation rates were determined by the quantitative collection of $^{14}CO_2$ produced (from TCA cycle activity) during the aerobic perfusion of the heart with Krebs'-Henseleit solution containing [1-^{14}C]palmitate as described previously (Lopaschuk et al., 1994; Lopaschuk and Barr, 1997). Briefly hearts were perfused in a closed recirculating system using an oxygenator with a large surface area in constant contact with 95%O_2 /5%CO_2. This ensures that the perfusate was adequately oxygenated. The gaseous mixture (including the $^{14}CO_2$ produced by the citric acid cycle and released into the perfusion chamber, see Figure 2-1) was then trapped by bubbling it through 1 M benzethonium hyamine hydroxide. Buffer sample (3 ml) and 0.25 ml of benzethonium hyamine hydroxide sample were removed at each time point to measure palmitate oxidation rates.

To extract $^{14}CO_2$ trapped in buffer samples, two 1 ml buffer samples were each injected into a 25 ml stoppered Erlynmeyer flask with filter paper saturated with 0.25ml of 1 M benzethonium hyamine hydroxide. Samples were acidified by contact with 9N H_2SO_4 (1 ml) already present in the flask to liberate $^{14}CO_2$ from the ^{14}C-labelled bicarbonate present in the buffer. The flask was shaken gently for 1 hour and the $^{14}CO_2$ trapped (by the hyamine hydroxide filter paper) was determined by placing the papers into a vial and adding 5 ml of ACS® scintillation cocktail. The amount of gaseous $^{14}CO_2$ trapped in the 0.25 ml of the benzethonium hyamine hydroxide sample and the amount of radiolabelled $^{14}CO_2$ present in the buffer samples were counted using standard β-scintillation counting procedures. Palmitate oxidation rate was expressed as nmol palmitate oxidized / min / total dry weight of the heart.

2-4 Tissue preparation:

At the end of the perfusion protocol described above, the frozen ventricular tissue was weighed and powdered using a pestle and mortar cooled to the temperature of liquid nitrogen. Powdered ventricular tissue was then transferred to cryovials and stored at –80°C until heart tissues were ready for analysis. Approximately 100 mg of powdered ventricular tissue was also used to determine the dry to wet ratio of the ventricles. This was used to determine the total dry weight of the ventricles which in addition to atrial dry weight was used to calculate the total dry weight of each of the perfused newborn heart.

2-5 Extraction of AMPK and ACC:

Tissue homogenization:

Approximately 200 mg of frozen ventricular tissue was homogenized in a buffer containing Tris/HCl (50 mM; pH 7.5 at 4° C), mannitol (250 mM), NaF (50 mM), Na-PPi (5 mM), EDTA (1 mM), EGTA (1 mM), dithiothreitol (DTT, 1 mM), and the following protease inhibitors: phenylmethylsulphonylfluoride (PMSF, 1 mM), soybean trypsin inhibitor (4 μg/ml) and benzamidine (1 mM). Samples were then centrifuged at 14,000 xg for 20 min at 4° C. The supernatant was brought to 2.5% polyethylene glycol (PEG) 8000 with a 25% (w/v) PEG 8000 stock, and the sample agitated for 10 min at 4° C. Samples were then spun at 10,000 xg for 10 min at 4° C. The supernatant was made up to 6% PEG 8000 using the PEG 8000 stocks and stirred once again for 10 min at 4° C. This fraction was spun at 10,000 xg for 10 min and the pellet was washed once with homogenization buffer containing 6% PEG 8000. This was followed by a final centrifugation at 10,000 xg for 10 min, and resuspension of the 6% PEG 8000 pellet in the resuspension buffer containing Tris/HCl (50 mM; pH 7.5 at 4° C), NaF (50 mM), NaPPi (5 mM), EDTA (1 mM), EGTA (1 mM), DTT, (1 mM), PMSF, (1 mM), soybean trypsin inhibitor (4 µg/ml) benzamidine (1 mM), glycerol (8% v/v) and sodium azide (0.4% v/v). The protein concentration of the extract was measured using a Sigma BCA protein kit.

2-6 *Enzyme assays:*

AMP-activated protein kinase assay:

AMPK activity was measured according to methods previously described by Kudo et al., (1995) and Kudo et al., (1996). Briefly, initial characterization of AMPK activity in newborn hearts were carried out by addition of varying amounts of protein in PEG 8000 extracts (2.5% and 6%) to the reaction mixture (25 µl final volume). This reaction mixture (pH 7.4) consist of a final concentration of HEPES-NaOH (40 mM), NaCl (80 mM), glycerol (8% v/v), EDTA (0.8 mM), SAMS peptide (200 μM), DTT (0.8 mM), [γ^{32}P]-ATP (200 μM), $MgCl_2$ (5 mM), and triton-X-100 (0.18 % v/v). Samples were also incubated in the presence or absence of 200 μM AMP. This mixture was then incubated for different time course at 30° C, and 15 μl sample was spotted on a 1 cm^2 phosphocellulose paper at the end of the reaction time. The paper was then washed 4 x 30 min with 150 mM phosphoric acid followed by a 20 min acetone wash at room temperature. Papers were then dried and put into a scintillation vial. ACS® scintillation cocktail (5 ml) was added and radioactivity was counted using standard scintillation counting techniques. AMPK activity was expressed as pmol ^{32}P incorporated into SAMS peptide / min / mg protein.

Acetyl CoA carboxylase assay:

ACC activity in PEG 8000 extracts (2.5% and 6%) was measured by the $^{14}CO_2$ fixation assay described previously by Kudo et al., (1995) and Kudo et al., (1996). Briefly, 5 μl of the PEG 8000 fraction containing 20 μg total protein, was added to a reaction mixture (final volume 165 μl) containing Tris acetate (60.6 μM, pH 7.4), bovine serum albumin (1 mg/ml), β-mercaptoethanol (1.32 μM), ATP (2.12 mM), acetyl CoA (1.06 mM), magnesium acetate (5 mM), [n-^{14}C]-$NaHCO_3$ (18.08 mM) and magnesium citrate (10 mM). Samples were then incubated at 37° C for 2 min, and the reaction was stopped by adding 25 μl of perchloric acid (10% v/v). Samples were spun for 20 min at 10,000 xg and 160 μl of supernatant was placed in minivials and dried in a fume hood overnight. 160 μl of H_2O was then added to the vials followed by scintillation counting. ACC activity was expressed as nmol $^{14}CO_2$ incorporated into malonyl CoA / min / mg protein.

Phosphorylation control of AMPK and ACC:

To gain an insight into the phosphorylation status of AMPK and ACC in the newborn heart, PEG 8000 extracts were prepared under conditions of maximal phosphorylation (when endogenous protein phosphatases were inhibited by addition of NaF (50 mM) and NaPPi (5 mM) in extraction buffers or under conditions of minimal phosphorylation (when NaF and NaPPi were omitted from the extraction buffers). In some studies, incubation of extracts were carried out either in the presence or absence of ATP (200 μM) and $MgCl_2$ (5 mM) or in the presence or absence of protein phosphatase 2A (PP2A, 15 mU/ml) or protein phosphatase 1C (PP1C, 5 U/ml). AMPK and ACC assays were then carried out as described above.

2-7 *Analysis of AMPK subunits and ACC isoenzymes:*

Electrophoresis:

To determine if AMPK was present in the newborn heart or if experimental conditions resulted in altered AMPK protein level, 6% PEG 8000 extracts from heart tissues were subjected to a 9% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970).

To detect if ACC was present in the fetal rabbit hearts or if experimental conditions changed ACC activity secondary to an altered ACC protein levels 6% PEG 8000 extract was subjected to a 5% SDS-PAGE. Ammonium persulphate was used to initiate gel polymerization and TEMED was used as catalysts for the reaction. Once the gel polymerized, the wells were rinsed briefly and 25 μg protein for AMPK and 40 μg protein for ACC were loaded per well. The gel was then run in a sample buffer containing 25 mM Tris, 190 mM glycine and 0.1%

SDS at 60 V for 1 hour to separate protein bands through the stacking gel. Voltage was then increased to 116 V until the dye front migrated to the end of the polyacrylamide gel.

Immunoblotting:

Following gel electrophoresis, the fractionated protein was transferred to nitrocellulose membrane using a wet transfer method. Briefly, the gel was removed and placed between nitrocellulose membrane and filter paper in a transfer buffer consisting of methanol (20% v/v), Tris (25 mM) and glycine (192 mM). The protein were then electroblotted to the nitrocellulose membrane at 100 V (constant voltage) for 1 hour at 4 ^{0}C. Membranes were then blocked with 10% w/v milk powder in phosphate buffered saline [PBS, consisting of NaCl (350 mM), KCl (3 mM), Na_2HPO_4 (10 mM) and KH_2PO_4 (2 mM)] for 4 hours at 4 ^{0}C. The membranes were washed for 2 hours at 4 ^{0}C with PBS containing 1% w/v milk powder and streptavidin-conjugated horseradish peroxidase (1:1000 v/v). Membranes were again washed 4 times with PBS containing 0.05% v/v Tween 20 and 3 times with PBS. Biotin containing ACC protein content of membrane was visualized by chemiluminescent detection method using ECL western blotting detection kit as previously described by Saddik et al., (1993). AMPK protein content was determined using polyclonal anti-rat liver AMPK α1 or α2 catalytic subunit primary antibody raised in sheep and kindly provided by Dr. Graham Hardie, Dundee, Scotland. Another polyclonal anti-AMPK catalytic subunit antibody from Dr. L.A Witters, Hanover, New Hampshire was used to detect AMPK on nitrocellulose membranes. Secondary peroxidase conjugated rabbit anti-sheep IgG was used to identify AMPK subunits. Chemiluminescent detection method was then performed to visualize AMPK protein by using an ECL western blotting detection kit followed by autoradiography on KODAK X-ray film.

2-8 *Densitometric analysis*

Following chemiluminescent detection, ACC and AMPK bands as seen on the autoradiograms were scanned using OFOTO® software package and images were acquired on a Power Macintosh computer. Following calibration of the optical density, images on the autoradiogram were analyzed on an image analysis program using NIH image® version 1.54 software to obtain optical density of each band [measured in arbitrary units (AU)]. Optical densities so obtained were then subjected to statistical analysis.

2-9 Determination of CoA esters and nucleotide levels using HPLC:

Determination of CoA esters levels:

200 mg portion of the powdered frozen ventricular tissue was used to prepare a 6% v/v perchloric acid (6% PCA) extract at 4 ^{0}C. Briefly, 1 ml of 6% PCA was put in a mortar, frozen ventricular tissues were added and the tissues were homogenized using a pestle until a slurry of fine consistency was obtained. The 6% PCA extract, maintained at 4 ^{0}C was then used to determine the levels of CoA esters on HPLC using internal standards as described previously by Saddik et al. (1993). Briefly, PCA extracts were centrifuged at 10,000 xg for 10 min and the supernatant was maintained at a pH of 2.5 (acid pH) using dithiothreitol (0.32 mM) and MOPS (1 M). The CoA esters present in extracts were measured using a modified HPLC technique originally described by King et al. (1988).

Determination of adenine nucleotide levels:

200 mg of frozen ventricular tissue was used to prepare 6% v/v PCA extract at 4 ^{0}C as described above. The 6% PCA extract was then used to determine adenine nucleotide levels by HPLC technique originally described by Park and Ally, (1992). Briefly, 6% PCA extract was transferred into a 10 ml test tube and was centrifuged at 10, 000 xg for 10 min. The supernatant was removed and 95 µl of dithiothreitol (0.32 mM) was added. The resulting supernatant was then neutralized with KOH (5 M) and centrifuged at 10, 000 xg for 10 min at room temperature to remove the resulting precipitate. The supernatant was analyzed for adenine nucleotides (ADP, AMP) and high energy phosphate (ATP) on HPLC by a modified HPLC method previously described by Ally and Park, (1992).[1]

2-10 *Determination of cyclic AMP levels:*

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[1] All tissue preparations and 6% perchloric acid extractions from samples were carried out by myself. Mr. Kenneth Strynadka was responsible for loading samples unto the HPLC and analyzing results.

200 mg of pulverized frozen heart tissue (obtained from control, glucagon and isoproterenol treated hearts) was homogenized in 10% v/v trichloroacetic acid in phosphate buffer [PBS, containing NaCl (80 mM), KCl (3 mM), Na_2HPO_4 (10 mM) and KH_2PO_4 (2 mM)] at 4 ^{0}C. The homogenate was then centrifuged at 1500 x g for 10 min. The supernatant was carefully removed into a 10 ml test-tube. The organic phase was subsequently removed by extraction in saturated diethyl ether:water (5:1 v/v). This extraction procedure was repeated three times. Any residual ether in aqueous phase was thereafter removed by gentle heating in a water bath set at 70 ^{0}C for 5 min. The aqueous phase containing the extracted cAMP was used to determine protein concentration of each sample by using BCA protein assay method (Sigma). The amount of cAMP present in each extract was determined by using an enzymatic cAMP colorimetric assay kit (Cayman Chemical Co). In these experiments, cAMP levels in heart tissues were expressed as µmol cAMP / g dry wt.

2-11 *Statistical analysis:*

Data are expressed as the mean ± standard error of the mean. Comparisons were performed using unpaired Student's t-test where appropriate. Where three or more group means were compared, analysis of variance followed by Neuman-Keuls test was used to determine statistical significance between groups. In some experiments, multivariate analysis of variance was used to determine differences between groups. Statistical level of significance was set at $p < 0.05$.

2-12 Materials:

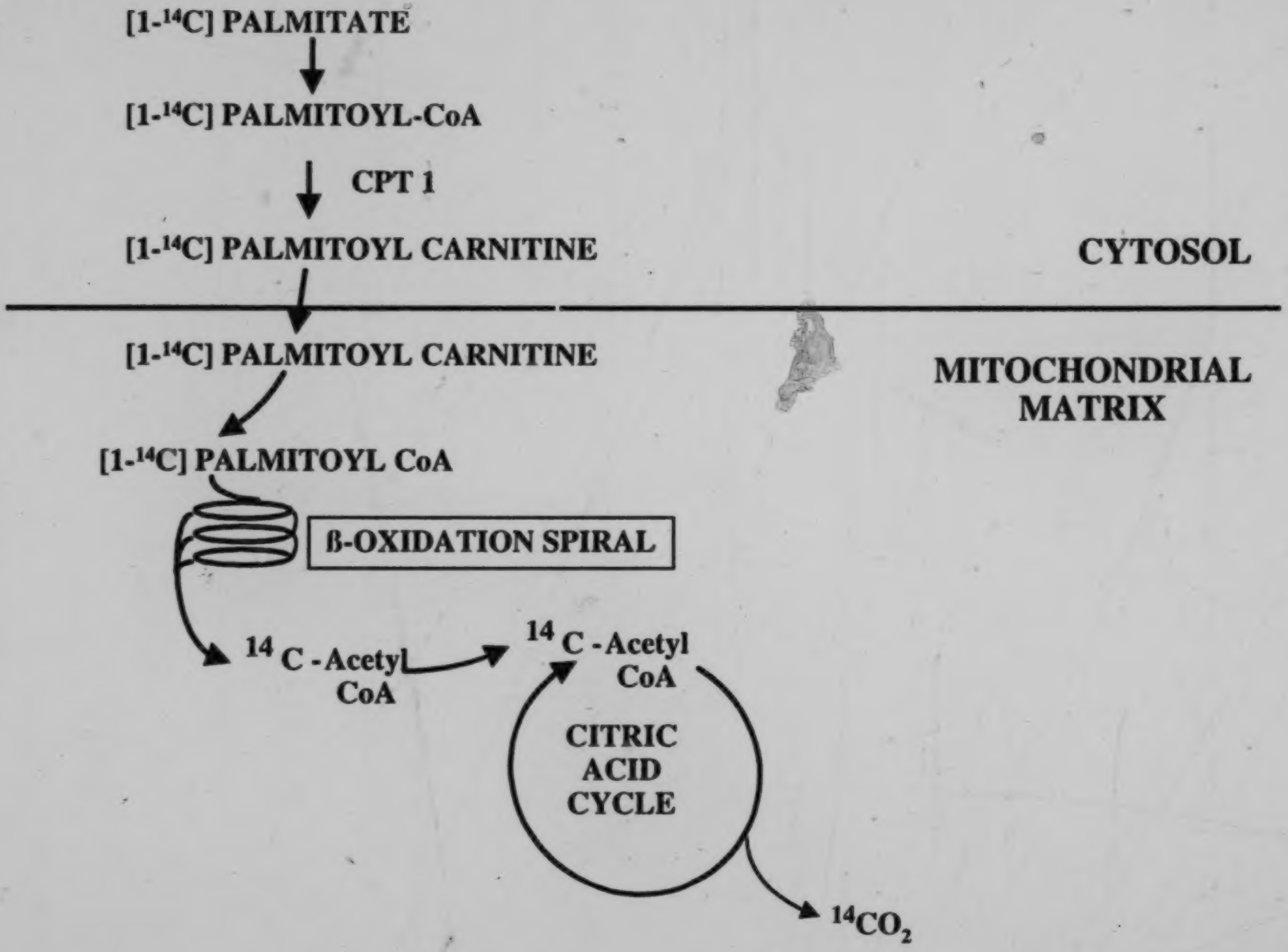
Acetyl CoA was obtained from Boehringer Mannheim. AMP, 2-Bromo AMP (sodium salt) and ATP (disodium salt) were obtained from Sigma Chemical Co. Insulin (Iletin® beef and pork insulin) was from Eli Lilly Canada (Toronto, Ontario). Glucagon (from porcine pancreas) was from Sigma Chemical Co. Bovine serum albumin, fraction V was from Boehringer Mannheim. The synthetic peptide substrate for measurement of AMPK activity with the amino acid sequence HMRSAMSGLHLVKRR (SAMS peptide) and AMARAASAAALARRR (AMARA peptide) were synthesized by the Alberta Peptide Institute, University of Alberta, Edmonton, Alberta. 5-amino-4-imidazolecarboxamide riboside (AICAR) and 2'deoxyadenosine were obtained from Sigma Chemical Co. Iodotubercidin was obtained from Research Biochemicals International (RBI) and Drs. Alan Patterson and Wendy Gati, Department of Pharmacology, University of Alberta. Polyclonal anti rat liver AMPK catalytic subunit antibody raised in sheep was a gift from Dr. D.G Hardie, Dundee, Scotland and Dr. L.A Witters, Hanover, New Hampshire, USA. γ–^{32}P-ATP as well as [n-^{14}C]bicarbonate and [1-^{14}C]palmitate were obtained from ICN

Radiopharmaceuticals. Benzethonium hyamine hydroxide was from NEN Chemicals. ACS® aqueous counting scintillant was from Amersham Canada (Oakville, Ontario). Streptavidin conjugated peroxidase was purchased from Mandel Scientific. Trans-Blot nitrocellulose membrane was from Bio-Rad. ECL western blotting detection reagents were purchased from Amersham International. BioMax MR biologic imaging X-ray films were purchased from Kodak, Rochester, N.Y. Sodium dodecylsulphate (SDS) was from BDH Biochemicals, USA. TEMED (electrophoresis grade) was from GIBCO Life Technologies, USA. All other chemicals were purchased from the Sigma Chemical Co.

Figure 2-1:

Fate of radiolabelled palmitate in the newborn heart.

[1-^{14}C]palmitate is taken up by the heart and activated to [1-^{14}C]palmitoyl CoA. This radiolabelled palmitoyl CoA is then converted into [1-^{14}C]palmitoylcarnitine by the enzyme carnitine palmitoyltransferase 1 (CPT 1). The radiolabelled palmitoylcarnitine enters the mitochondria where it releases the [1-^{14}C]palmitoyl CoA moiety which then passes through the β-oxidation spiral with the formation of [1-^{14}C]acetyl CoA. The [1-^{14}C]acetyl CoA then enters the citric acid cycle (also known as tricarboxylic acid cycle) where $^{14}CO_2$ is released during citric acid cycle activity (see Lopaschuk and Barr, 1997). Quantitative collection of the $^{14}CO_2$ released during heart perfusion was used as a measure of palmitate oxidation rate.


[1-14C] PALMITATE
[1-14C] PALMITOYL-CoA
CPT 1
[1-14C] PALMITOYL CARNITINE
CYTOSOL
[1-14C] PALMITOYL CARNITINE
MITOCHONDRIAL MATRIX
[1-14C] PALMITOYL CoA
ß-OXIDATION SPIRAL
14 C -Acety CoA
14 C -Acetyl CoA
CITRIC ACID CYCLE
14CO2

References:

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Barr, R.L., and Lopaschuk, G.D. (1997) Direct measurement of energy metabolism in the isolated working rat heart. *J. Pharmacol.& Toxicol. Methods* **38,** 11-17.

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Kudo, N., Barr, A.J., Barr, R.L., Desai, S., and Lopaschuk, G.D. (1995) High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl CoA levels due to an increase in 5'AMP-activated protein kinase inhibition of acetyl CoA carboxylase. *J. Biol. Chem.* **270**, 17513-17520.

Kudo, N., Gillespie, J.G., Kung, L., Witters, L.A., Schulz, R., Clanachan, A.S., and Lopaschuk, G.D. (1996) Characterization of 5'AMP-activated protein kinase in the heart and its role in inhibiting acetyl CoA carboxylase during reperfusion following ischemia. *Biochim. Biophys. Acta* **1301**, 67-75.

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Lopaschuk, G.D., and Barr, R.L. (1997), Measurement of fatty acid and carbohydrate metabolism in the isolated working rat heart. *Mol. Cell. Biochem.* **172,** 137-147.

Lopaschuk, G.D., Witters, L.A, Itoi, T., Barr, R., and Barr, A. (1994) Acetyl CoA carboxylase involvement in the rapid maturation of fatty acid oxidation in the newborn rabbit heart. *J. Biol. Chem.* **269**, 25871-25878.

Saddik, M., Gamble, J., Witters, L.A., and Lopaschuk, G.D. (1993) Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *J. Biol. Chem.* **268,** 25836-25845.

CHAPTER 3

Characterization of 5'AMP-activated protein kinase in fetal and newborn rabbit hearts

Introduction

Fatty acid oxidation increases dramatically in 7-day compared with 1-day old rabbit heart (Lopaschuk and Spafford, 1990). Although the reason for this increase in fatty acid oxidation remains to be fully elucidated, recent studies in rat heart demonstrate that acetyl CoA carboxylase (ACC) regulates fatty acid oxidation in the heart via its production of malonyl CoA (Saddik et al., 1993; Lopaschuk and Gamble, 1994). Malonyl CoA itself is a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1), the rate limiting enzyme in the mitochondrial uptake of activated fatty acids (McGarry et al., 1978; McGarry et al., 1983). Following birth, ACC activity and malonyl CoA levels decrease in 7-day compared with 1-day old heart (Lopaschuk et al., 1994). This decrease in ACC activity is responsible for the increase in fatty acid oxidation in 7-day old heart. To date, the reason for the decrease in ACC activity in 7-day old heart remains unknown.

Two isoforms of ACC (280 kDa and 265 kDa) have been demonstrated to be present in newborn heart (Lopaschuk et al., 1994). Studies by Davies et al. (1990), however, demonstrate that rat liver ACC can be phosphorylated and inactivated by a novel AMP-activated protein kinase (AMPK). AMPK has since been characterized in different tissues and cell lines as shown in Table 3-1. A candidate kinase responsible for the decreased ACC activity in newborn heart (as in liver) may, therefore, be AMPK. AMPK is a multisubstrate protein kinase consisting of α catalytic and β, γ regulatory subunits (Carling et al., 1990; Woods et al., 1996). More recent studies by Hawley et al., (1996) have shown that AMPK itself is under phosphorylation control by an upstream kinase kinase (termed AMPK kinase or AMPKK). Whether AMPK is expressed in the newborn heart is not known. In addition, whether AMPK in the newborn heart can actually phosphorylate and inactivate newborn heart ACC remains to be determined. We therefore hypothesized that newborn heart contains an active AMPK that can phosphorylate and inactivate ACC. (Figure 3-1).

The main purpose of studies presented in this chapter is to characterize AMPK in newborn rabbit heart. It is further the objective of this study to determine if indeed AMPK in the newborn heart was present prior to birth or possibly, if the AMPK gene was activated in the immediate newborn period. We demonstrate here that AMPK is present in newborn rabbit heart.

Experimental Procedures:

Materials:

2-Bromo-AMP (Br-AMP, sodium salt) was obtained from Sigma Chemical Co. The synthetic peptide substrates for the measurement of AMPK activity with the amino acid sequences HMRSAMSGLHLVKRR (SAMS peptide) and AMARAASAAALARRR (AMARA peptide) were synthesized by the Alberta Peptide Institute, University of Alberta, Edmonton, Alberta, Canada. All other chemicals and reagents were obtained as described in Methods.

Extraction of fetal and newborn hearts:

Hearts from fetal (29 days gestation), 1-day and 7-day old New Zealand white rabbits (either sex) were used in this study. Hearts were extracted as described in Methods. Hearts (unperfused) were quickly rinsed in Krebs-Henseleit solution and frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen and stored immediately at –80 ^{0}C until ready for analysis. Unless otherwise stated, characterization of AMPK activity was carried out using 7-day old hearts.

Tissue preparations and extraction of AMPK and ACC:

All tissue preparations were carried out as described in Methods. Polyethylene glycol 8000 extraction of frozen ventricular tissue was carried out to obtain AMPK and ACC proteins as described in Methods.

Western blot analysis of AMPK and ACC in fetal and newborn hearts:

To determine if the newborn rabbit heart contains AMPK, 7-day old hearts were used to prepare PEG 8000 extracts as in Methods. These extracts (post-mitochondria, 2.5% and 6% PEG) were then subjected to 9% SDS-PAGE. To determine if AMPK was present prior to birth, fetal and 1-day old heart tissues were used to prepare 6% PEG 8000 extracts. These extracts were then subjected to 9% SDS-PAGE. AMPK catalytic and regulatory subunit abundance were determined using polyclonal anti AMPK $\alpha 2$-subunit antibody and anti-AMPK β

or γ subunit antibodies (dilution of 1:5000 v/v) as described in Methods. Densitometric analysis of AMPK bands following autoradiography were carried out as described in Methods. ACC protein contents of both fetal and 1 day old hearts were also determined using 5% SDS-PAGE as described in Methods.

Measurement of AMP-activated protein kinase and acetyl CoA carboxylase activity:

AMPK assay:

Since previous characterization of AMPK in the newborn heart has not been done, a time course and protein dependence of AMPK activity in these hearts were determined. Briefly, AMPK activity was determined in the presence or absence of 200 μM AMP using 1, 2, 4, 6, 8, or 10 μg protein from 6 % PEG 8000 extracts. AMPK activity was also determined following 1, 2, 4, or 6 min incubation periods in the presence or absence of Br-AMP (a cell permeable but inactive AMP analogue) and in the presence of increasing concentrations of AMP. Moreover, AMPK activity in post-mitochondria, 2.5% and 6% PEG 8000 extracts was determined using either SAMS peptide or AMARA peptide.

To determine the phosphorylation status of AMPK and its effect on AMPK activity, 50 mM sodium fluoride and 5 mM sodium pyrophosphate (phosphatase inhibitors) were either included or omitted from both homogenization buffer and resuspension buffers (collectively termed extraction buffers) to allow for minimal or maximal activation of endogenous protein phosphatase activity, respectively, during the extraction protocol. AMPK activity in 6% PEG 8000 extracts obtained in the presence or absence of sodium fluoride and sodium pyrophosphate was then determined. In another series of experiments, AMPK activity was determined in 6% PEG 8000 extracts with or without pre-incubation of the extract with 5 mM $MgCl_2$ and 200 μM ATP (ATP/Mg^{2+}) for either 0 or 30 min. Also pre-incubation of 6% PEG 8000 extracts was carried out with or without protein phosphatase 2A (PP2A, 15 U/ml) or protein phosphatase 1C (PP1C, 5U/ml) followed by determination of AMPK activity.

ACC assay:

In order to determine whether changes in AMPK activity were associated with alterations in ACC activity, 6% PEG 8000 extracts obtained in the presence or absence of sodium fluoride and sodium pyrophosphate were used to measure ACC activity as described in Methods.

Statistical analysis:

Data are expressed as the mean ± standard error of the mean. Comparisons were performed using unpaired Student's t-test where appropriate. Where three or more group means were compared, analysis of variance followed by Neuman-Keuls test was used to determine significant difference between groups. Statistical level of significance was set at $p < 0.05$.

Results:

AMPK and ACC proteins in fetal and newborn rabbit hearts:

Shown in Figure 3-2 is the presence of AMPK protein in post-mitochondria, 2.5% PEG 8000 and 6% PEG 8000 extracts from 7-day old hearts. Figure 3-2A shows a band corresponding to the highly purified rat liver AMPK on a commassie blue stained gel. This band was confirmed to be AMPK by Western immunoblot analysis using polyclonal anti-AMPK $\alpha2$ catalytic subunit antibody (Figure 3-2B). Densitometric analysis of bands showed that AMPK abundances in all three PEG 8000 extracts were not significantly different. (Figure 3-3).

Shown in Figures 3-4 is the presence of AMPK β (A) and γ (B) regulatory subunits, respectively, in 7-day old hearts. Although the β subunit abundances in post-mitochondria, 2.5% PEG 8000 and 6% PEG 8000 extracts were not different, densitometric analysis revealed that there is a higher abundance of AMPK γ subunit in the 6% PEG 8000 extract (Figure 3-5). Interestingly, Western blot analysis revealed that AMPK was present prior to birth in the 29-day gestation fetal rabbit heart (Figure 3-6A). Moreover, as shown in Figure 3-6B, ACC protein can be detected in both fetal and 1-day old rabbit hearts. It is interesting to note that whereas fetal heart contains only the 280 kDa isoform of ACC, 1-day old hearts contain both 280 kDa and 265 kDa ACC isozymes.

AMPK activity in fetal and newborn hearts:

The fact that AMPK immunoprotein can be detected in both fetal and newborn rabbit heart, however, does not necessarily confirm that AMPK is in its catalytically active form. As shown in Figure 3-7, AMPK activity increased with increasing amount of protein in the assay medium. AMPK activity was maximal with the addition of 4 μg protein. Shown in Figure 3-8 is the AMPK activity with increasing time of reaction in the assay medium. AMPK activity was maximal at 4 min incubation. Addition of 200 μM AMP to the assay medium increased AMPK activity by approximately two-fold (Figure 3-8). Addition of 200 μM Br-AMP, however, had no significant effect on AMPK activity (Figure 3-8). When AMPK activity was determined in the presence of increasing concentrations of AMP using 2 μg protein in the assay medium for 3 min, AMPK activity was maximal at 200 μM AMP (Figure 3-9). Determination of AMPK activity in the presence or absence of 200 μM AMP in both fetal and 1-day old hearts however, revealed that activity was higher in fetal hearts (Figure 3-10).

To confirm that the kinase activity measured was indeed that of AMPK, PEG 8000 extracts from 7-day old heart (post-mitochondria supernatant, 2.5% and 6% PEG 8000) were used to measure AMPK activity using either SAMS or AMARA peptides. Shown in Table 3-2 is AMPK activity in these extracts. AMPK activity was highest in 6% PEG 8000 extracts regardless of whether SAMS or AMARA peptide was used as substrate. This result is consistent with previously published studies which also showed that the majority of AMPK activity is present in the 6% PEG 8000 extract (Carling et al., 1990; Kudo et al., 1995; Kudo et al., 1996).

Phosphorylation control of AMPK activity in newborn rabbit hearts:

To gain an insight into the phosphorylation status of AMPK in PEG 8000 extracts and its effect on AMPK activity, PEG 8000 extraction was carried out in the presence or absence of sodium fluoride and sodium pyrophosphate in the extraction buffer. AMPK activity was higher when sodium fluoride and sodium pyrophosphate were present in the extraction buffer (Figure 3-11). When the 6%

PEG 8000 extract was pre-incubated with ATP/Mg^{2+} for a 30 min pre-incubation period, AMPK activity increased compared to controls (Figure 3-12). Moreover, pre-incubation of 6 % PEG 8000 extracts with PP2A or PP1C for 30 min resulted in a significant decrease in AMPK activity (Figure 3-13).

When ACC activity was determined in 6% PEG 8000 extracts obtained in the presence or absence of sodium fluoride and sodium pyrophosphate in the extraction buffer, ACC activity increased in the absence of sodium fluoride and sodium pyrophosphate compared with that obtained in presence of protein phosphatase inhibitors (Figure 3-14). These data suggest that AMPK in the newborn heart may be under phosphorylation control.

Discussion:

Results from this study demonstrate that the newborn rabbit heart contains AMPK with measurable activity. As shown in Figures 3-2 and 3-4 both α catalytic subunit and regulatory β and γ subunits of AMPK can be detected by Western immunoblot analysis of PEG 8000 extracts obtained from 7-day old heart. Although the nature of subunit association is yet to be fully elucidated, our data are in agreement with previously published studies by Stapleton et al., (1994), Woods et al., (1996) and Dyck et al., (1996) that demonstrate essentially that all three subunits of AMPK (α, β, and γ) are important for catalytic activity.

Another novel finding arising from the present study is that AMPK is actually expressed prior to birth in the rabbit heart (Figure 3-6A). In fact, there is a higher abundance of AMPK protein in the fetal rabbit heart compared to 1-day old heart. The reason for a high AMPK abundance in the fetal rabbit heart is as yet unknown. Since the fetal heart is, however, dependent on carbohydrate metabolism as a source of high energy phosphates and AMPK may be involved in regulating carbohydrate metabolism, it is conceivable that AMPK probably plays a role to regulate carbohydrate metabolism in the fetal rabbit heart.

It is of interest that ACC 280 is expressed in the rabbit heart prior to birth in the 29-day gestation fetal rabbit heart (Figure 3.6B). This suggests that ACC 280 is the catalytically more important ACC isozyme responsible for malonyl CoA production in the heart. These data therefore confirm our earlier observation that both isozymes of ACC (ACC 265 and 280 KDa) are present in the immediate newborn period (Lopaschuk et al., 1994).

Studies by other investigators (Table 3-1) demonstrate that AMPK activity is measurable in various tissues including hepatocytes, adipocytes and rat liver homogenates. It is, however, not known if AMPK exists in a catalytically active form in newborn rabbit heart. Using SAMS peptide or AMARA peptide (both are peptide substrates for AMPK) we showed that [^{32}P-γ]-ATP incorporation into SAMS peptide was highest in 6% PEG 8000 extracts from 7-day old heart when compared to 2.5% PEG 8000 extracts or post-mitochondria supernatant. This observation was reproduced when AMPK activity was measured using AMARA peptide (see Table 3.2). A serendipitous observation arising from the use of these two peptides is that AMPK activity measured in 6% PEG 8000 extract was higher when AMARA peptide was used as peptide substrate compared with activity measured with SAMS peptide.

Although the reason for high AMPK activity in 6% PEG 8000 extracts using AMARA peptide remains unclear, it has been previously reported that AMARA peptide was a better peptide substrate for AMPK compared to SAMS peptide (Dale et al., 1995). This is probably due to the ease of phosphorylation of the peptide by AMPK. It is also possible that 6% PEG 8000 extracts contain a higher amount of AMPK Kinase which can phosphorylate and activate AMPK. Indeed Hardie and coworkers recently characterized AMPK kinase in rat liver and showed that 6% PEG 8000 extracts contain a considerable amount of AMPK kinase activity (Weekes et al., 1994; Hawley et al., 1996). The similarity in the pattern of AMPK activity seen with both peptides therefore serves, both to validate our assay as well as to demonstrate that AMPK activity is highest in 6% PEG 8000 extracts. Another possible reason may be related to increased extraction of γ subunit of AMPK in 6% PEG 8000 extracts (see Figure 3-4B).

To confirm further the validity of our assay and to determine the effect of AMP (an allosteric activator) on AMPK activity, we determined the effect of 200 μM AMP and 200 μM Br-AMP on AMPK activity. As can be seen from Figures

3-8 and 3-9, 200 μM AMP resulted in a further activation of AMPK activity compared to controls whereas addition of 200 μM Br-AMP did not cause an activation of AMPK activity (Figure 3-8). As the name of this kinase therefore suggests, AMPK is activated in the presence of micromolar concentrations of AMP and should not be confused with cAMP dependent protein kinase (PKA).

Another finding from this study is that newborn rabbit heart AMPK appears to be under phosphorylation/dephosphorylation control by kinases and phosphatases. Indeed by three lines of evidence we demonstrate that phosphorylation of AMPK results in an increased AMPK activity and that its dephosphorylation results in decreased AMPK activity. First, as shown in Figure 3-11, AMPK activity increased when 6% PEG 8000 extraction was carried out in the presence of phosphatase inhibitors (sodium fluoride and sodium pyrophosphate), suggesting that increased phosphatase activity results in a dephosphorylation and inactivation of AMPK. Second, when PEG 8000 extracts were pre-incubated with PP2A or PP1C, AMPK activity was almost completely abolished (see Figure 3-13). Third, pre-incubation of the 6% PEG 8000 extracts (obtained with the inclusion of phosphatase inhibitors in the extraction buffer) with ATP/Mg^{2+} resulted in increased AMPK activity. These data therefore confirm that phosphorylation results in increased AMPK activity whereas dephosphorylation decreases AMPK activity. These results are consistent with a previously published report by Hardie and coworkers which showed that AMPK is highly susceptible to phosphorylation by kinases and dephosphorylation by protein phosphatase 2A or 2Cα (Davies et al., 1995; Moore et al., 1991). Although direct evidence is still lacking, this reversible phosphorylation of AMPK may represent a major mechanism by which AMPK activity is regulated in vivo in the newborn rabbit heart.

Another finding from this study was that an increased AMPK activity appears to be associated with a decreased ACC activity and vice-versa. When PEG 8000 extracts were obtained in the presence of sodium fluoride and sodium pyrophosphate, AMPK activity was higher and ACC activity was less (Figure 3-11 and Figure 3-14) compared with AMPK and ACC activities obtained in the absence of these protein phosphatase inhibitors. It is, therefore, possible that in vivo, AMPK may directly regulate ACC activity.

Altogether we demonstrate that the newborn rabbit heart expresses both the α catalytic subunit and β and γ regulatory subunits of AMPK. We further showed that newborn heart AMPK is catalytically active. Moreover, newborn heart AMPK is subject to phosphorylation and dephosphorylation control by kinases and phosphatases, respectively. Since increased AMPK activity is associated with a decreased ACC activity and ACC is an important regulator of fatty acid oxidation (Saddik et al., 1993; Lopaschuk and Gamble, 1994), AMPK may regulate fatty acid oxidation secondary to an altered ACC activity in newborn heart. A proposed scheme by which AMPK regulation of ACC may result in altered fatty acid oxidation is shown in Figure 3-15. Further studies to clarify the involvement of AMPK in the regulation of fatty acid oxidation in newborn hearts are necessary.

Table 3-1:

Selected studies of AMP-activated protein kinase in various tissues and cell cultures.

Reference	Tissue /cell studied	Comments
Davies et al., 1992.	Rat liver	AMPK inactivates ACC in rat liver
Witters et al., 1992.	Hepatocytes	Insulin inhibits AMPK in hepatocytes
Sullivan et al., 1994.	Rat adipocytes	AICAR activates AMPK in rat adipocytes
Winder et al., 1997.	Skeletal muscle	Electrical stimulation of skeletal muscle increased AMPK activity
Dyck et al., 1996.	COS 7 cells	β and γ subunits of AMPK important for activity
Carling et al., 1990.	Rat liver	Rat liver AMPK purified and characterized
Kudo et al., 1996.	Rat heart	Characterized AMPK in rat heart

Table 3-2:

AMPK activity measured in PEG 8000 extracts from unperfused 7-day old rabbit hearts using SAMS peptide or AMARA peptide as the synthetic peptide substrate.

	AMPK activity (pmol / min / mg protein)		
Experimental condition	**Post-mitochondria supernatant**	**2.5% PEG**	**6% PEG**
SAMS peptide			
0 AMP	236 ± 18	170 ± 16	668 ± 28
200 µM AMP	208 ± 14	265 ± 20*	1285 ± 55*
AMARA peptide			
0 AMP	228 ± 22	254 ± 11	755 ± 12
200 µM AMP	248 ± 25	525 ± 24*	1693 ± 32*

Values are the mean ± SEM of AMPK activity measurements from 6 different 7-day old rabbit hearts using SAMS peptide or AMARA peptide.

*** significantly different from 0 AMP ; PEG = Polyethylene glycol 8000**

Figure 3.1:

Possible mechanism by which activation of AMPK results in a decreased ACC activity and malonyl CoA levels in the newborn rabbit heart.

AMPK kinase may phosphorylate AMPK with a resultant increase in AMPK activity. The activated AMPK then phosphorylates and inactivates ACC. Malonyl CoA (product of ACC activity) levels decreases under this condition.

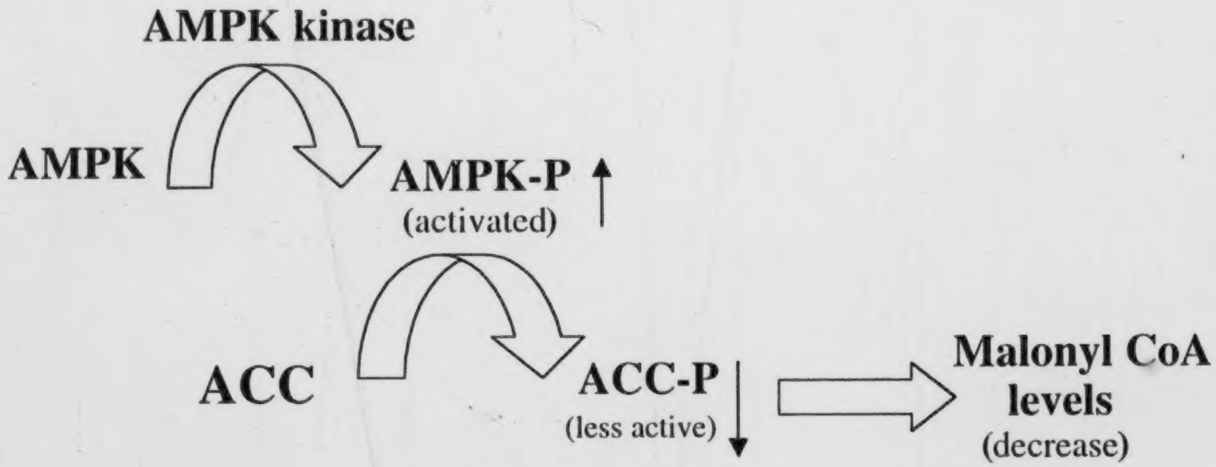

AMPK kinase
AMPK
AMPK-P ↑
(activated)
ACC
ACC-P ↓
(less active)
Malonyl CoA levels
(decrease)

Figure 3-2:

Representative (A) Commassie blue stained gel of PEG 8000 extracts from unperfused 7-day old hearts and purified rat liver AMPK following 9% SDS-PAGE. (B) Immunoblot analysis of newborn rabbit heart AMPK in PEG 8000 extracts and purified rat liver AMPK.

Lanes 1 & 2 = post-mitochondria supernatant; 3 & 4= 2.5% PEG; X = empty; S = highly purified rat liver AMPK; 5 & 6 = 6% PEG.

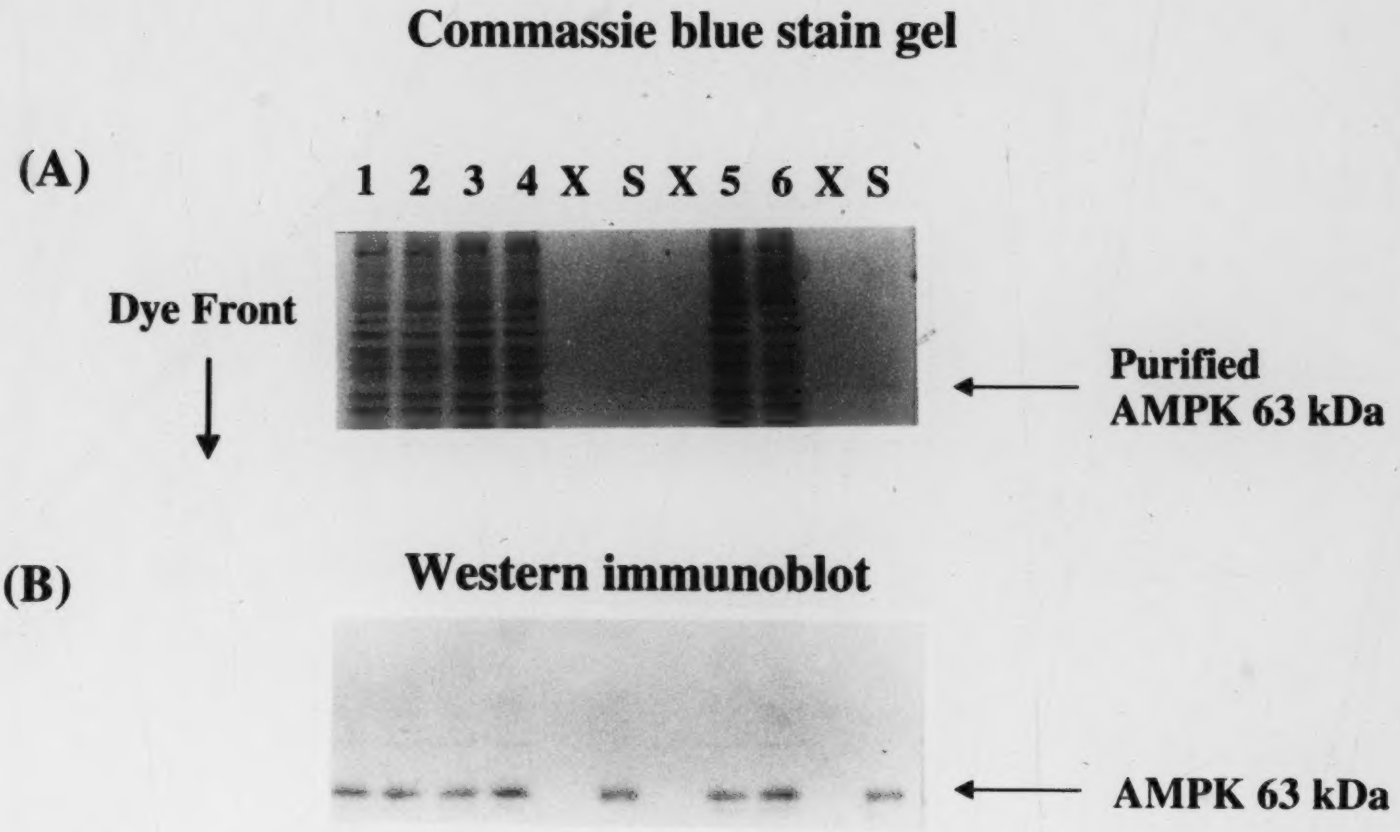

Commassie blue stain gel
(A)
1 2 3 4 X S X 5 6 X S
Dye Front
Purified
AMPK 63 kDa
(B)
Western immunoblot
AMPK 63 kDa

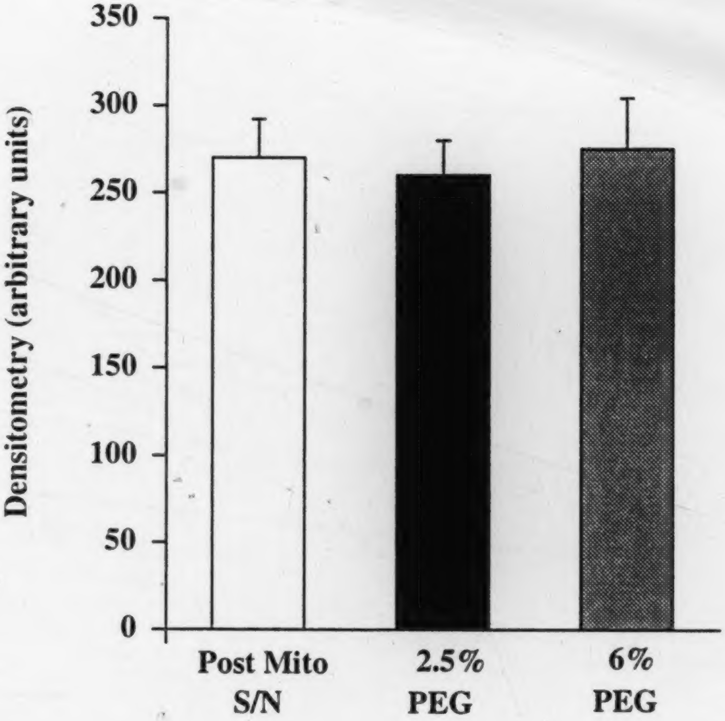
Figure 3-3:

Densitometric analysis of AMPK α2-subunit band intensity in PEG 8000 extracts obtained from unperfused 7-day old hearts.

Densitometric analysis was carried out as described in Methods.

Values are the mean ± S.E.M. of results from 6 different hearts.

Post Mito S/N = post-mitochondrial supernatant; 2.5% PEG = 2.5% polyethylene glycol 8000 extract; 6% PEG = 6% polyethylene glycol 8000 extract.



Densitometry (arbitrary units)
350
300
250
200
150
100
50
0
Post Mito S/N
2.5% PEG
6% PEG

Figure 3-4:

Representative immunoblot analysis of (A) β subunit and (B) γ subunit of AMPK in PEG 8000 extracts obtained from unperfused 7-day old hearts.

Western blot analysis of representative PEG 8000 fractions obtained from newborn rabbit heart was performed as described in Methods. AMPK β and γ regulatory subunits were visualized by using anti-AMPK β and γ regulatory subunit antibodies, respectively.

Lanes 1 & 2 = post-mitochondria supernatant; 3 & 4 = 2.5% PEG; 5 & 6 = 6% PEG.

Western immunoblot

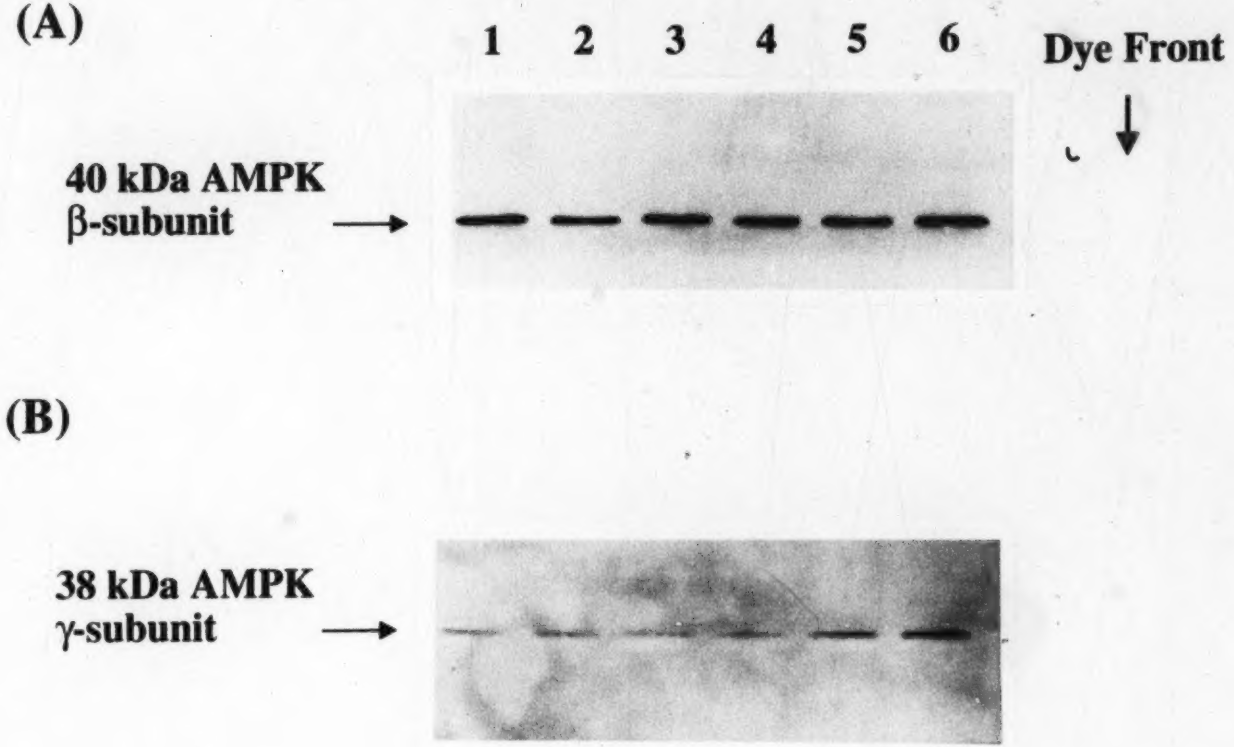

(A)
1
2
3
4
5
6
Dye Front
40 kDa AMPK
β-subunit
(B)
38 kDa AMPK
γ-subunit

Figure 3-5:

Densitometric analysis of AMPK γ-subunit band intensity in PEG 8000 extracts obtained from unperfused 7-day old hearts.

Densitometric analysis was carried out as described in Methods.

Values are the mean ± S.E.M. of results from 6 different hearts.

* significantly different from other groups shown by analysis of variance followed by Neuman-Keul's test.

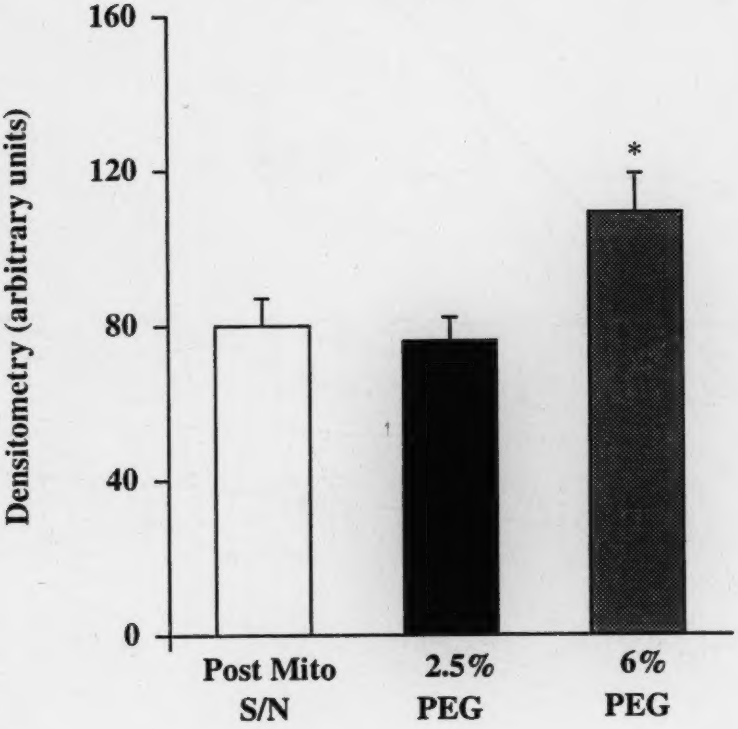

Densitometry (arbitrary units)
160
120
80
40
0
*
Post Mito S/N
2.5% PEG
6% PEG

Figure 3-6:

Representative immunoblot analysis of (A) AMPK (B) ACC in 6% PEG 8000 extracts obtained from unperfused fetal and 1-day old rabbit hearts.

Western blot analysis of representative 6 % PEG 8000 extracts obtained from fetal and 1-day old hearts were performed as described in Methods. AMPK α2 subunit was visualized by using anti-AMPK α2 subunit antibody.

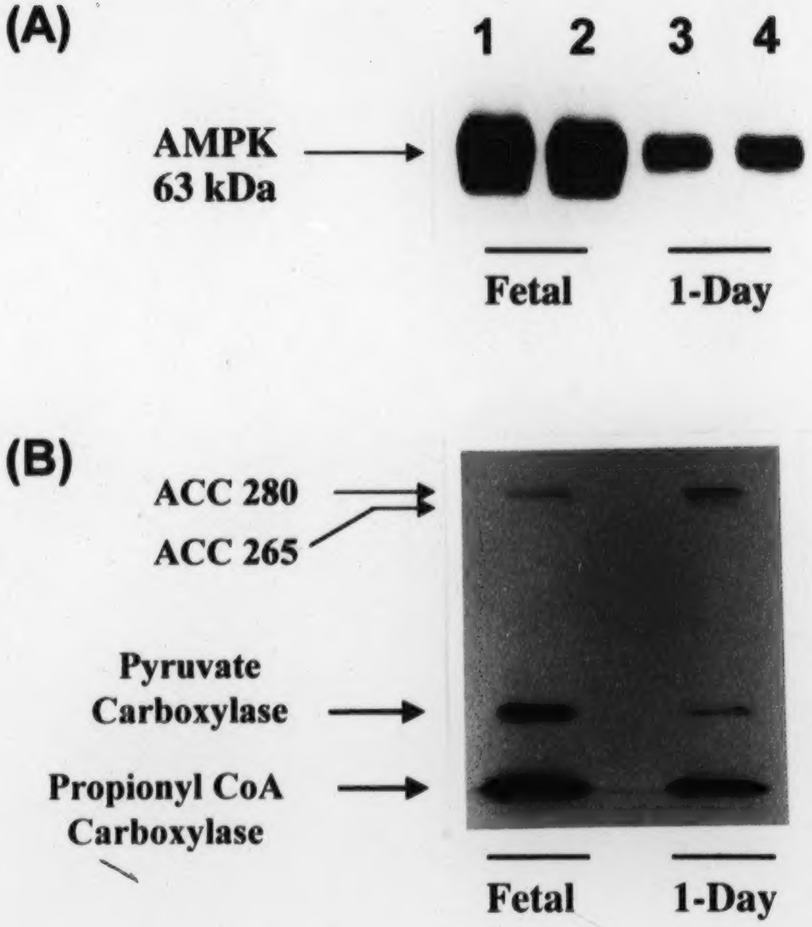

(A)
1
2
3
4
AMPK
63 kDa
Fetal
1-Day
(B)
ACC 280
ACC 265
Pyruvate
Carboxylase
Propionyl CoA
Carboxylase
Fetal
1-Day

Figure 3-7:

Effect of increasing amount of protein on AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts.

AMPK activity was determined in 6% PEG 8000 extracts in the presence or absence of 200 μM AMP and in the presence of increasing amount of protein. Values are the mean ± S.E.M. of results from 6 hearts.

* significantly different from 0 AMP by unpaired Student's 't' test.

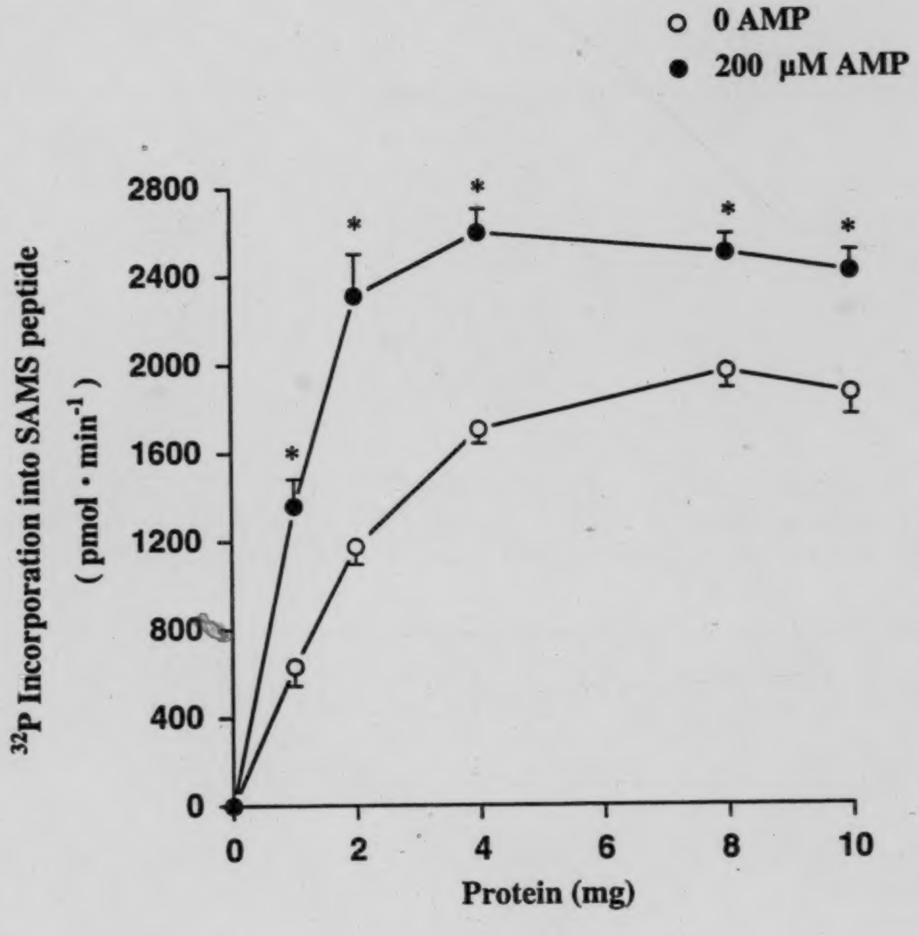

○ 0 AMP
● 200 μM AMP
^{32}P Incorporation into SAMS peptide
(pmol · min^{-1})
2800
2400
2000
1600
1200
800
400
0
*
*
*
*
*
0
2
4
6
8
10
Protein (mg)

Figure 3-8:

Time dependence of AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts.

AMPK activity was determined in 6% PEG 8000 extracts in the presence or absence of 200 μM AMP or 200 μM Br-AMP as described in Methods.

Values are the mean ± S.E.M. of results from 6 hearts.

* significantly different from 0 AMP by analysis of variance followed by Neuman-Keul's test.

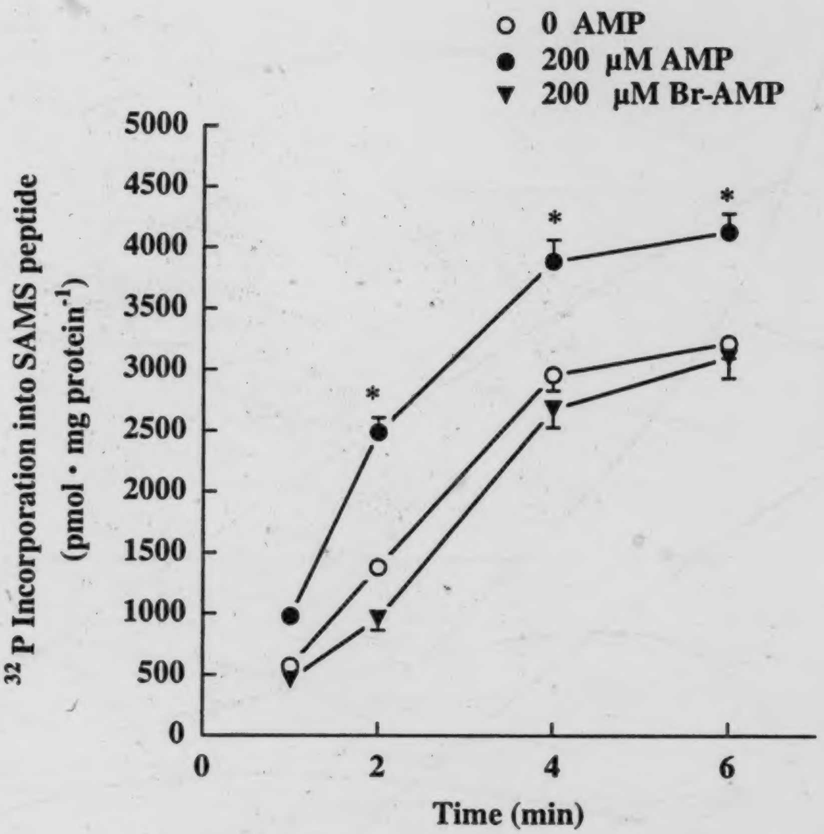

○ 0 AMP
● 200 µM AMP
▼ 200 µM Br-AMP
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
^{32}P Incorporation into SAMS peptide
(pmol • mg protein^{-1})
*
*
*
0
2
4
6
Time (min)

Figure 3-9:

Effect of increasing AMP concentration on AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts.

AMPK activity was determined in 6% PEG 8000 extracts in the presence of increasing concentrations of AMP.

Values are the mean ± S.E.M. of results from 6 hearts.

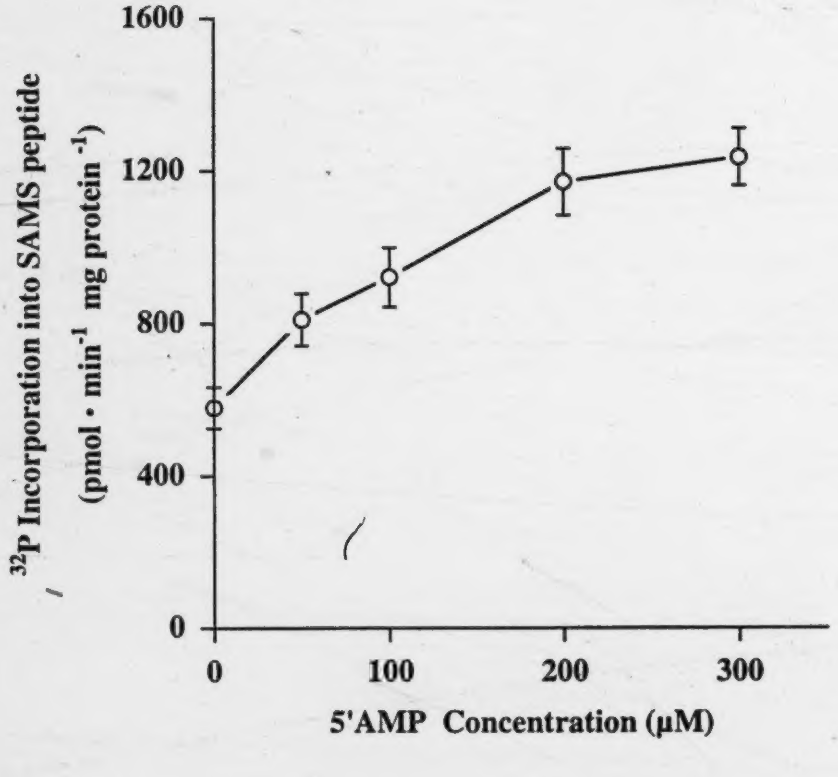

^{32}P Incorporation into SAMS peptide
(pmol · min^{-1} mg protein $^{-1}$)
1600
1200
800
400
0
0
100
200
300
5'AMP Concentration (µM)

Figure 3-10:

AMPK activity in unperfused fetal and 1-day old hearts.

AMPK activity was determined in 6% PEG 8000 extracts obtained from fetal and 1-day old hearts. AMPK activity was measured in the presence or absence of 200 µM AMP. Values are the mean ± S.E.M. of results from 8 fetal and 6 1-day old hearts.

* significantly different from 0 AMP by unpaired Student's 't' test.

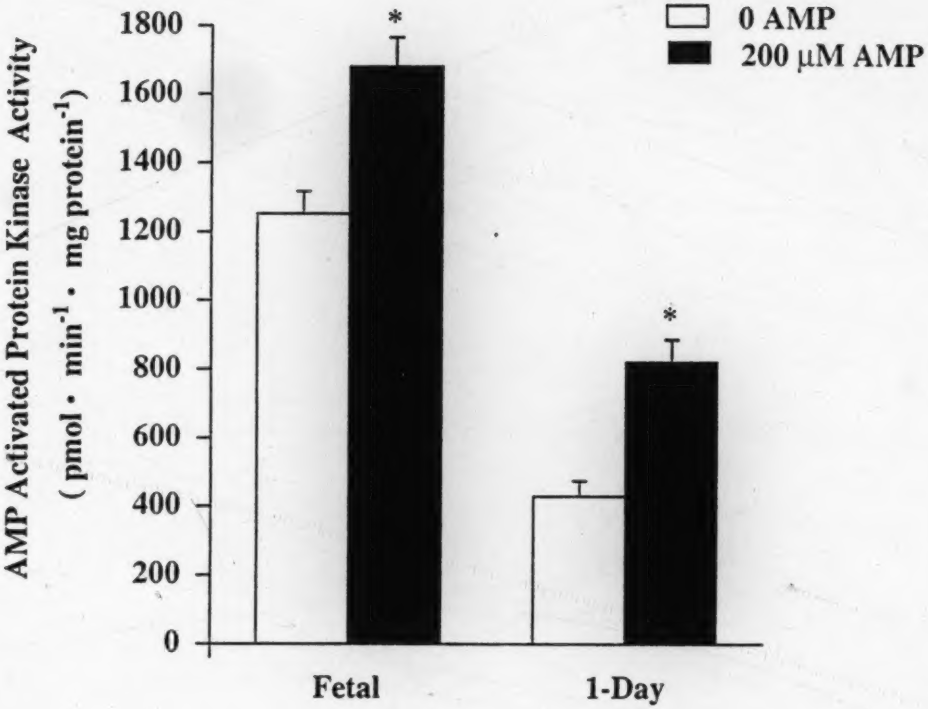

AMP Activated Protein Kinase Activity
(pmol • min^{-1} • mg protein^{-1})
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
Fetal
1-Day
☐ 0 AMP
■ 200 μM AMP

Figure 3-11:

AMPK activity in 6% PEG 8000 extracts from unperfused 7-day old hearts obtained in the presence or absence of sodium fluoride and sodium pyrophosphate in the extraction buffer.

AMPK activity was determined in 6% PEG 8000 extracts. AMPK activity was measured in the presence or absence of 200 µM AMP as described in Methods. Values are the mean ± S.E.M. of results from 6 different hearts.

* significantly different from 0 AMP by unpaired Student's 't' test.

† significantly different from results in the presence of sodium fluoride and sodium pyrophosphate by analysis of variance followed by Neuman-Keul's test.

NaF = sodium fluoride; NaPPi = sodium pyrophosphate.

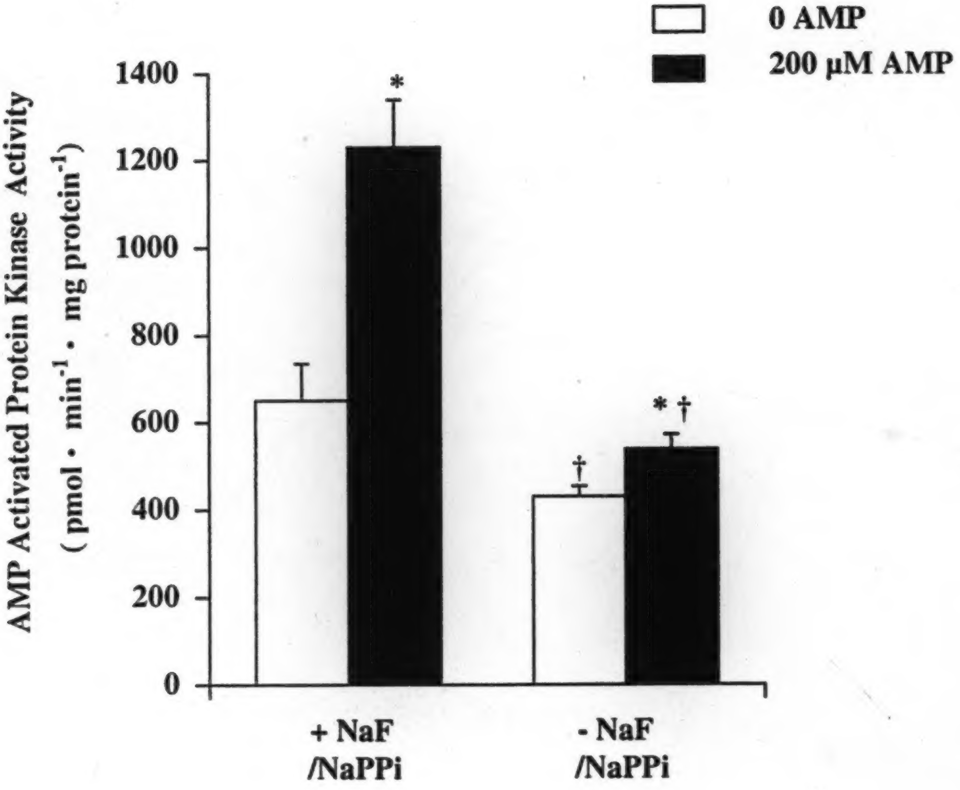

0 AMP
200 µM AMP
AMP Activated Protein Kinase Activity
(pmol • min^{-1} • mg protein^{-1})
1400
1200
1000
800
600
400
200
0
*
†
* †
+ NaF
/NaPPi
- NaF
/NaPPi

Figure 3-12:

AMPK activity following 30 min pre-incubation of 6% PEG 8000 extracts obtained from unperfused 7-day old heart with ATP /Mg^{2+}

AMPK activity was determined in 6% PEG 8000 extracts. AMPK activity was measured in the presence or absence of 200 µM AMP as described in Methods. Values are the mean ± S.E.M. of results from 6 different hearts.

* significantly different from 0 AMP by unpaired Student's 't' test.

† significantly different from no pre-incubation control by analysis of variance followed by Neuman-Keul's test.

a significantly different from 30 min control pre-incubation by Student's 't' test.

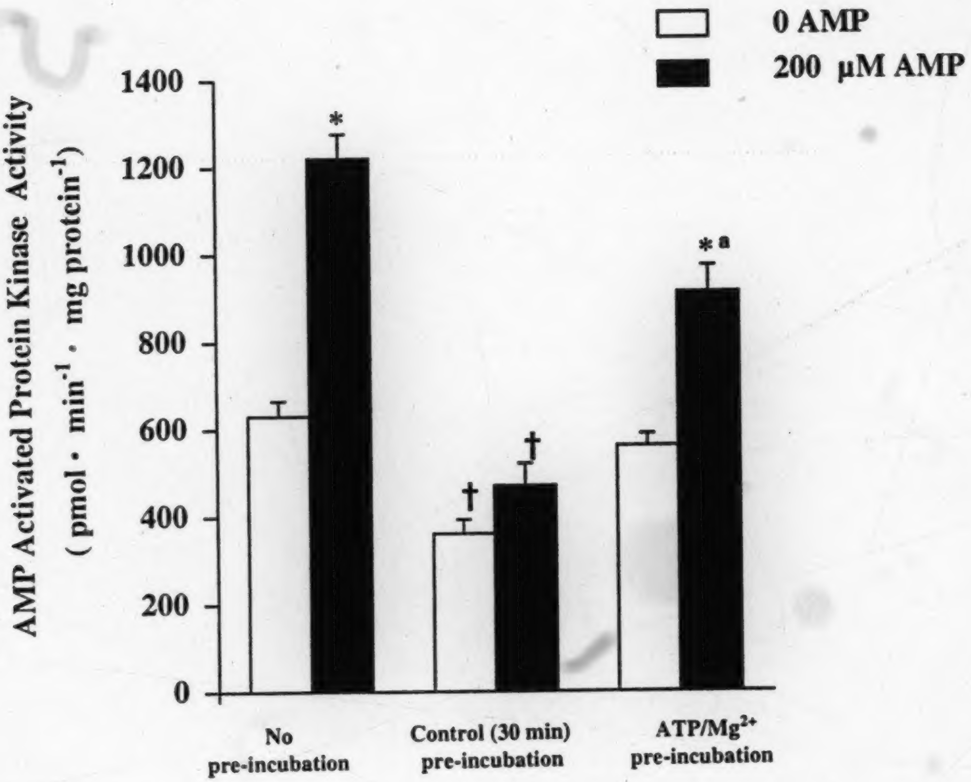

0 AMP
200 µM AMP
AMP Activated Protein Kinase Activity
(pmol · min^{-1} · mg protein^{-1})
1400
1200
1000
800
600
400
200
0
*
†
†
* a
No
pre-incubation
Control (30 min)
pre-incubation
ATP/Mg^{2+}
pre-incubation

Figure 3-13:

AMPK activity following 30 min pre-incubation of 6% PEG 8000 extracts from unperfused 7-day old hearts with PP2A or PP1C.

AMPK activity was determined in 6% PEG 8000 extracts. AMPK activity was measured in the presence or absence of 200 μM AMP as described in Methods. Values are the mean ± S.E.M. of results from 6 different hearts.

* significantly different from 0 AMP by unpaired Student's 't' test.

† significantly different from no pre-incubation control by analysis of variance followed by Neuman-Keul's test.

a significantly different from control (30 min) pre-incubation by analysis of variance followed by Neuman-Keul's test.

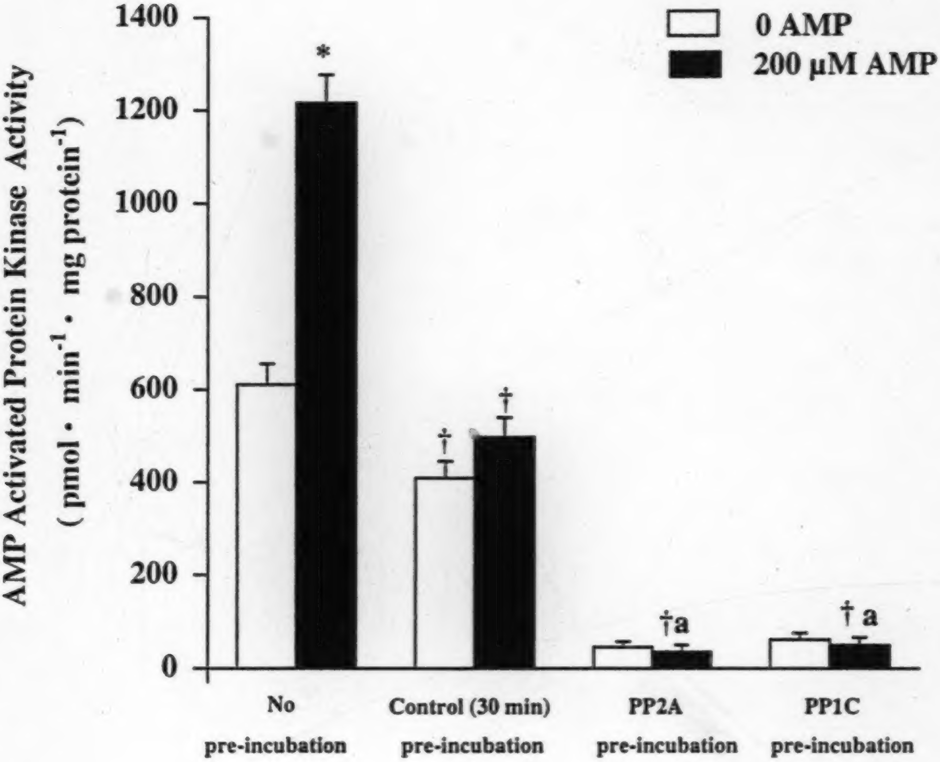

AMP Activated Protein Kinase Activity
(pmol • min^{-1} • mg protein^{-1})
1400
1200
1000
800
600
400
200
0
0 AMP
200 μM AMP
*
†
†
†a
† a
No
pre-incubation
Control (30 min)
pre-incubation
PP2A
pre-incubation
PP1C
pre-incubation

Figure 3-14:

ACC activity in 6% PEG 8000 extracts from unperfused 7-day old hearts obtained in the presence or absence of sodium fluoride and sodium pyrophosphate in the extraction buffer.

ACC activity was measured by $^{14}CO_2$ fixation assay as described in Methods. Values are the mean ± S.E.M. of results from 6 different hearts.

* significantly different from results obtained in the presence of sodium fluoride and sodium pyrophosphate by unpaired Student's 't' test.

NaF = sodium fluoride; NaPPi = sodium pyrophosphate.

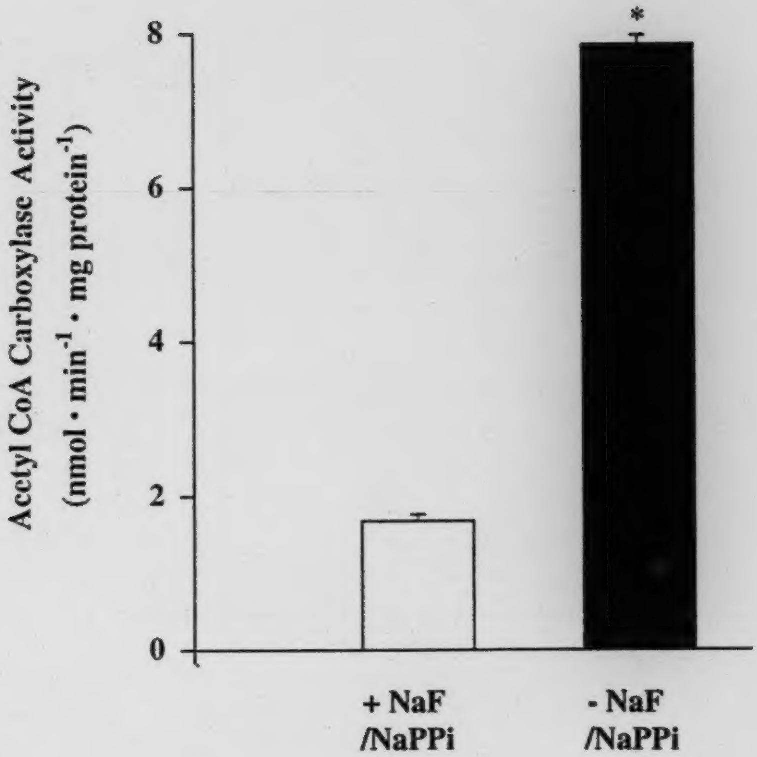
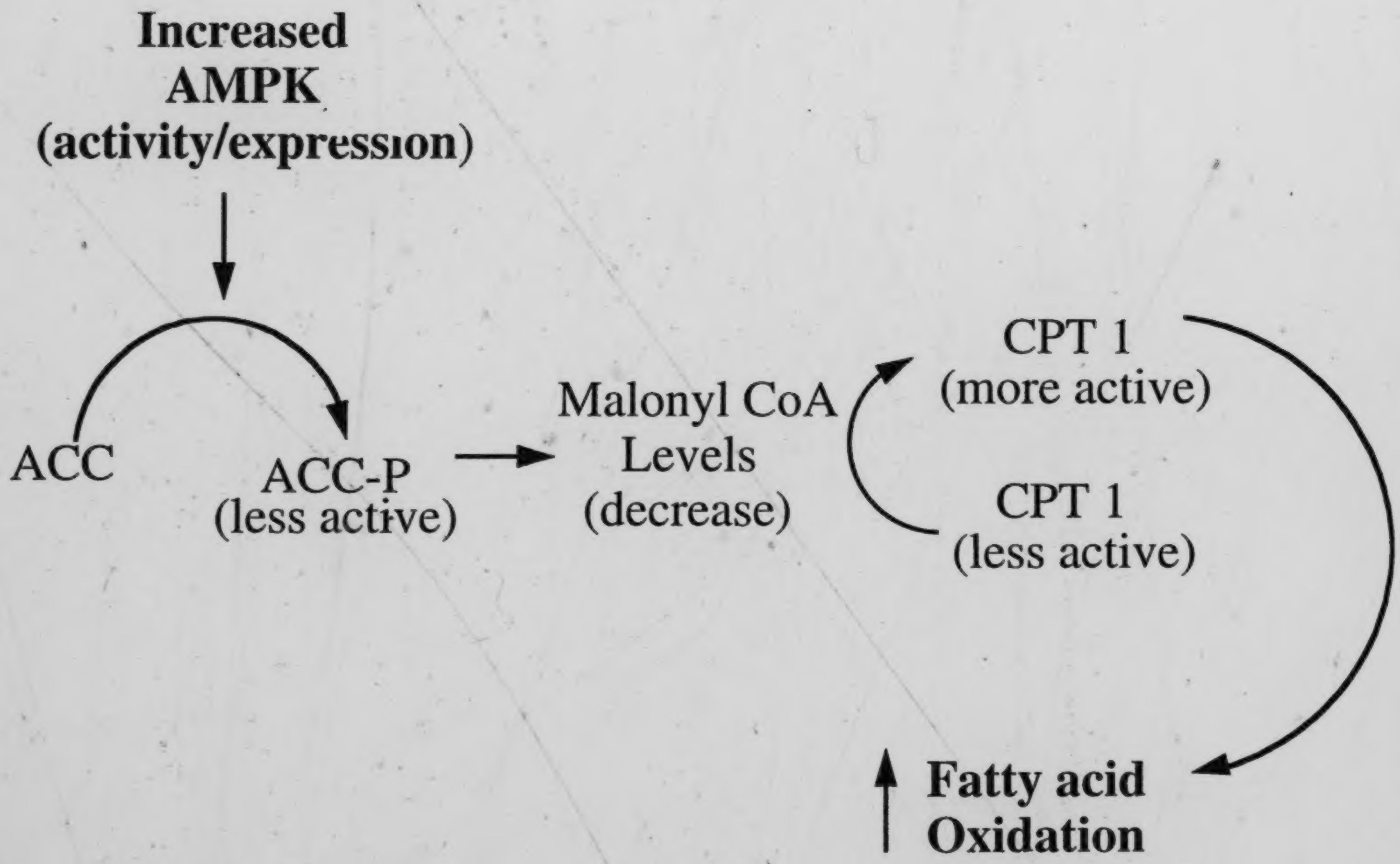

Acetyl CoA Carboxylase Activity
(nmol · min⁻¹ · mg protein⁻¹)
8
6
4
2
0
*
+ NaF /NaPPi
- NaF /NaPPi

Figure 3-15:

Proposed scheme showing the mechanism by which increased AMPK activity results in an increased fatty acid oxidation in newborn rabbit heart.

Increased AMPK catalytic activity and /or abundance in the immediate newborn period results in a decreased ACC activity and malonyl CoA levels. Malonyl CoA is a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1). The decreased levels of malonyl CoA relieve the inhibitory effects of malonyl CoA on CPT 1. The resultant increase in CPT 1 activity increases the uptake of activated fatty acid into the mitochondria for increased β-oxidation of fatty acids.


Increased
AMPK
(activity/expression)
ACC
ACC-P
(less active)
Malonyl CoA
Levels
(decrease)
CPT 1
(more active)
CPT 1
(less active)
↑ Fatty acid
Oxidation

References:

Carling, D., Clarke, P.R., Zammit, V.A., and Hardie, D.G. (1990) Purification and characterization of the AMP-activated protein kinase. *Eur. J. Biochem.* **186,** 129-136.

Dale, S., Wilson, W.A., Edelman, A.M., and Hardie, D.G. (1995) Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase 1. *FEBS Lett.* **361,** 191-195.

Davies, S.P., Carling, D., Munday, M.R., and Hardie, D.G. (1992) Diurnal rhythm of phosphorylation of rat liver acetyl–CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. *Eur. J. Biochem.* **203,** 615-623.

Davies, S.P., Helps, N.R., Cohen, P.T.W., and Hardie, D.G. (1995) 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phospatase-2Cα and native bovine protein phosphatase-2A. *FEBS Lett.* **377,** 412-425.

Davies, S.P., Sim, A.T.R., and Hardie, D.G. (1990) Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. *Eur. J. Biochem.* **187,** 183-190.

Dyck, J.R.B., Gao, G., Widmer, J., Stapleton, D., Fernandez, C.S., Kemp, B.E., and Witters, L.A. (1996) Regulation of 5'AMP activated protein kinase activity by the non-catalytic β and γ subunits. *J. Biol. Chem.* **271,** 17798-17803.

Hawley, S.A., Davidson, M., Woods, A., Davies, S.P., Beri, R.J., Carling, D., and Hardie, DG. (1996) Characterization of the AMP activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem.* **271,** 27879-27887.

Kudo, N., Barr, A.J., Barr, R.L., Desai, S., and Lopaschuk, G.D. (1995) High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl CoA levels due to an increase in 5'AMP-activated protein kinase inhibition of acetyl CoA carboxylase. *J. Biol. Chem.* **270,** 17513-17520.

Kudo, N., Gillespie, J.G., Kung, L., Witters, L.A., Schulz, R., Clanachan, A.S., and Lopaschuk, G.D. (1996) Characterization of 5'AMP-activated protein kinase in the heart and its role in inhibiting acetyl CoA carboxylase during reperfusion following ischemia. *Biochim. Biophys. Acta* **1301,** 67-75.

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McGarry, J.D., Leatherman, G.F., and Foster, D.W. (1978) Carnitine palmitoyltransferase 1: The site of inhibition of hepatic fatty acid oxidation by malonyl CoA. *J. Biol. Chem.* **253,** 4128-4136.

McGarry, J.D., Mills, S.E., Long, C.S., and Foster, D.W. (1983) Observations on the affinity for carnitine and malonyl CoA sensitivity of carnitine palmitoyltransferase 1 in animal and human tissues. *Biochem. J.* **214,** 21-28.

Moore, F., Weekes, J., and Hardie, D.G. (1991) Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. *Eur. J. Biochem.* **199,** 691-697.

Saddik, M., Gamble, J., Witters, L.A., and Lopaschuk, G.D. (1993) Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *J. Biol. Chem.* **268,** 25836-25845.

Stapleton, D., Gao, G., Michell, B.J., Widmer, J., Mitchelhill, K., Thh, T., House, C.M., Witters, L.A., and Kemp, B.E. (1994) Mammalian 5'AMP-activated protein kinase non-catalytic subunits are homologs of proteins that interact with yeast Snf1 protein kinase. *J. Biol. Chem.* **269,** 29343-29346.

Sullivan, J.E., Brocklehurst, K.J., Marley, A.E., Carey, F., Carling, D., and Beri, R.J. (1994) Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. *FEBS Lett.* **353,** 33-36.

Weekes, J., Hawley, S.A., Corton, J., Shugar, D., and Hardie, D.G. (1994) Activation of rat liver AMP-activated protein kinase by kinase kinase in a purified reconstituted system. *Eur. J. Biochem.* **219,** 751-757.

Winder, W.W., Wilson, H.A., Hardie, D.G., Rasmussen, B.B., Hutber, C.A., Call, G.B., Clayton, R.D., Conley, L.M., Yoon, S., and Zhou, B. (1997) Phosphorylation of rat muscle acetyl CoA carboxylase by AMP-activated protein kinase and protein kinase *Am. J. Appl. Physiol.* **82,** 219-225.

Witters, L.A., and Kemp, B.E. (1992) Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J. Biol. Chem.* **267,** 2864-2867.

Woods, A., Cheung, P.C., Smith, F.C., Davison, M.D., Scott, J., Beri, R.K., and Carling, D. (1996) Characterization of AMP activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J. Biol. Chem.* **271,** 10282-10290.

Chapter 4

Upregulation of 5'AMP-activated protein kinase is responsible for the increase in myocardial fatty acid oxidation following birth in newborn rabbit

Introduction

Previous studies in our laboratory have demonstrated that the newborn heart contains an active 5'AMP-activated protein kinase (AMPK) which is involved in the regulation of acetyl CoA carboxylase (ACC). Following birth, ACC activity decreases precipitously in 7-day compared to 1-day old rabbit heart. This decrease in ACC activity in 7-day old heart is accompanied by a dramatic increase in fatty acid oxidation as energy substrate such that fatty acids become a major source of energy for the developing heart (Lopaschuk and Spafford, 1990; Lopaschuk et al., 1991; Lopaschuk et al., 1994). The role of AMPK in the regulation of ACC activity and fatty acid oxidation in 7-day old heart remains unknown. We hypothesized that an increased AMPK abundance and/or activity is responsible for the decreased ACC activity and subsequent increase in fatty acid oxidation in 7-day old heart.

ACC is an important regulator of fatty acid oxidation via its production of malonyl CoA (Saddik et al., 1993; Lopaschuk and Gamble, 1994). We recently demonstrated that malonyl CoA levels decreased significantly in 7-day old heart compared to 1-day old heart (Lopaschuk et al., 1994). This decrease in malonyl

CoA levels results in increased carnitine palmitoyltransferase (CPT) 1 activity, a key enzyme involved in fatty acid uptake by mitochondria (McGarry and Foster, 1980). This results in a significant increase in fatty acid oxidation in 7-day compared to 1-day old heart.

In 1-day old hearts, ACC activity is markedly stimulated by insulin (Lopaschuk et al, 1994) and is accompanied by increased malonyl CoA levels. This has potential significance in the maturation of fatty acid oxidation since in the immediate newborn period, circulating levels of insulin decrease dramatically (Girard et al., 1992). As a result, a decrease in insulin has the potential to decrease ACC activity and lower malonyl CoA levels. Recent studies in hepatocytes demonstrate that insulin can stimulate ACC activity via inhibition of AMPK (Witters and Kemp, 1992). Whether insulin inhibits newborn heart AMPK to increase ACC activity and decrease fatty acid oxidation is not known. In addition, it has yet to be determined if stimulation of AMPK activity contributes to the increased fatty acid oxidation observed in newborn hearts.

The purpose of this study is to determine if an increase in AMPK activity could explain the decreased ACC activity and the increase in fatty acid oxidation in 7-day old heart. We demonstrate that heart AMPK is upregulated following birth in 7-day old heart.

Experimental Procedures:

Materials:

Acetyl CoA (trilithium salt) was obtained from Boehringer Mannheim. SAMS peptide used in the AMPK assays was synthesized by the Alberta Peptide Institute. Anti-AMPK catalytic subunit antibody was a gift from Dr. L.A. Witters, Hanover, NH. USA. All other chemicals and materials were obtained as described in Methods.

Heart perfusions:

One and 7-day old New Zealand white rabbit hearts were used in this study. Hearts were either unperfused as described in Methods or perfused in the Langendorff condition as described in Methods. Hearts were perfused with Krebs-Henseleit solution containing 11 mM glucose and 0.4 mM [1-^{14}C]palmitate bound to 3% bovine serum albumin in the presence or absence of insulin (100 μU/ml), or in the presence or absence of 5-amino 4-imidazolecarboxamide riboside (AICAR, 200 μM). Perfusate was delivered to the heart at a 60 mm Hg pressure. Steady state palmitate oxidation rates were measured during a 40 min aerobic perfusion period as described in Methods.

Tissue work-up:

Following heart perfusions, ventricles were quickly frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen as described in Methods. PEG 8000 extracts were then prepared from pulverized frozen ventricular tissue as described in Methods.

Measurement of AMP-activated protein kinase and acetyl CoA carboxylase activity:

AMPK assay:

AMPK activity in PEG 8000 extracts obtained from 1 and 7-day old hearts perfused with and without insulin were determined as described in Methods. To determine if AMPK from 1-day old heart is subject to phosphorylation /dephosphorylation control, 6% PEG 8000 extracts obtained from 1 and 7-day old hearts were pre-incubated with 200 μM ATP and 5 mM $MgCl_2$ (ATP/Mg^{2+}) for 30 min thereafter AMPK activity was determined as described in Methods.

ACC assay:

ACC activity in 6% PEG 8000 extracts obtained from 1 and 7-day old hearts perfused with and without insulin were determined as described in Methods.

Western blot analysis of AMPK:

Samples of 6% PEG 8000 extracts from unperfused 1 and 7-day old rabbits were subjected to a 9% SDS-PAGE as described in Methods. Following gel electrophoresis, AMPK protein abundance was determined using anti-AMPK catalytic subunit polyclonal antibody kindly provided by Dr. L.A. Witters, Hanover, N.H, USA. Chemiluminescent detection was performed followed by autoradiography as described in Methods.

Statistical analysis:

Data are expressed as the mean ± standard error of the mean. Comparisons between groups were performed using the unpaired Student's t-test. Where appropriate, analysis of variance followed by the Neuman-Keul's test was used to determine significant differences when more than two group means were compared. Statistical significance was set at $p < 0.05$.

Results:

AMPK activity and abundance in newborn hearts:

Shown in Figure 4-1 is AMPK activity measured in 1-day and 7-day unperfused rabbit hearts. Basal AMPK activity was higher in 7-day hearts compared to 1-day hearts. Addition of 200 µM AMP to the incubation medium increased AMPK activity in both 1-day and 7-day hearts (Figure 4-1). Shown in Figure 4-2 is the AMPK catalytic subunit abundance in unperfused hearts from 1-day and 7-day old rabbits. AMPK was detected in both age groups, however, AMPK abundance was higher in 7-day compared to 1-day rabbit hearts.

Palmitate oxidation rates and acetyl CoA carboxylase activity in 1-day and 7-day old hearts:

Shown in Figure 4-3 is the effect of insulin on palmitate oxidation in isolated Langendorff perfused hearts from 1-day and 7-day old rabbit hearts. In the absence of insulin, the rate of palmitate oxidation was less in 1-day old hearts compared to 7-day old hearts. Addition of insulin (100 µU/ml) resulted in a significant decrease in rate of palmitate oxidation in 1-day old hearts, but was without any significant effect in 7-day old hearts. Under these conditions, rate of palmitate oxidation was almost 4-fold higher in 7-day old hearts compared to 1-day old hearts. The increase in the rate of palmitate oxidation in 7-day old hearts was accompanied by a decrease in ACC activity compared to 1-day old heart (Figure 4-4).

Effect of insulin on AMPK activity in newborn rabbit hearts:

AMPK activities in 1-day old and 7-day old hearts perfused in the presence and absence of insulin (100 μU/ml) are shown in Figure 4-5. In the absence of insulin, AMPK activity was significantly higher in 7-day old rabbit hearts compared to 1-day old hearts. Addition of insulin to the perfusate decreased AMPK activity in both 1-day old and 7-day old hearts. If 6% PEG 8000 extracts from perfused hearts were pre-incubated with protein phosphatase 2A (in the absence of sodium fluoride/sodium pyrophosphate) a significant decrease in AMPK activity was seen in all groups. (Figure 4-6).

Phosphorylation control of AMPK in the newborn heart:

Table 4-1 shows AMPK activities following pre-incubation with ATP/Mg^{2+} for 30 min in 6% PEG 8000 extracts obtained from 1-day and 7-day old hearts perfused in the presence or absence of insulin. AMPK activity was significantly lower in control pre-incubation compared to no pre-incubation. If 200 μM ATP and 5 mM $MgCl_2$ were present during pre-incubation, the dramatic decrease in AMPK activity was abolished. In fact, an increase in AMPK activity was observed compared to control pre-incubation (Table 4-1). This confirms that AMPK is under phosphorylation control in both 1-day and 7-day old hearts.

Effect of activation of AMPK on ACC activity and fatty acid oxidation:

Recent studies in hepatocytes and adipocytes have shown that 5-amino 4-imidazolecarboxamide riboside (AICAR) can stimulate AMPK activity (Corton et al., 1995). To investigate the effect of direct stimulation of AMPK on ACC and fatty acid oxidation, we perfused a series of 7-day old rabbit hearts with AICAR (200 μM). As shown in Table 4-2, perfusion of hearts with AICAR resulted in a significant increase in AMPK activity compared with controls. Accompanying this increase in AMPK activity, there was a significant decrease in ACC activity and a significant increase in rate of palmitate oxidation.

Discussion:

We demonstrate in this study that AMPK is not only present in the newborn heart, but the abundance and activity of AMPK increase after birth. Western immunoblot analysis revealed that AMPK abundance was higher in 7-day old hearts compared to 1-day old hearts. This was accompanied by a greater AMPK activity in 7-day old hearts under basal conditions i.e. no insulin in the perfusate (see Figures 4-1 and 4-2). It is therefore possible that increased AMPK activity results in an increased phosphorylation and inactivation of ACC.

The importance of ACC in the regulation of fatty acid metabolism in the adult and newborn heart has recently been established (Saddik et al., 1993; Lopaschuk and Gamble, 1994; Kudo et al., 1995; Kudo et al., 1996). The mechanism responsible for regulating ACC in the heart, however, remains poorly understood. Previous studies in other tissues (liver and adipose tissues) have shown that ACC is under the control of a novel cytoplasmic enzyme, AMPK (Davies et al., 1990; see Hardie, 1992 for review). As demonstrated in this study, newborn heart contains an active AMPK that we hypothesized is actively involved in regulating newborn heart ACC.

ACC activity and malonyl CoA levels decreased in 7-day old heart which was accompanied by an increase in fatty acid oxidation (Lopaschuk et al., 1994). In this study, we demonstrate that, in the presence of insulin, ACC activity increased and both fatty acid oxidation and AMPK activity decreased in 1-day old hearts compared to 7-day old hearts (see Figures 4-3; 4-4 and 4-5). Removal of insulin from the perfusate resulted in a significant decrease in ACC activity and was accompanied by a significant increase in AMPK activity. It is of interest that in 7-day old hearts, AMPK activity was significantly higher than in 1-day old hearts. A decreased ACC activity and an increased fatty acid oxidation accompanied this increase in AMPK activity in 7-day old heart. Since insulin levels are low seven days post-birth (Girard et al., 1992), these results suggest that hormonal control of AMPK by insulin may be an important regulator of fatty acid oxidation in the immediate newborn period. We suggest that the decrease in circulating insulin levels seen following birth (Girard et al., 1992), results in an activation of AMPK and a decrease in ACC activity. The resulting decrease in malonyl CoA levels (Lopaschuk et al., 1994) results in an increase in fatty acid oxidation. A proposed scheme describing this is shown in Figure 4-7.

Further evidence supporting a role for AMPK in regulating fatty acid oxidation is provided by our AICAR results. AICAR is a cell permeable activator of AMPK, which is taken up by the cell and converted to 5-amino-4-imidazolecarboximide ribotide (ZMP) (Corton et al., 1995). Recent studies in liver and adipose tissue have shown that ZMP activates AMPK and decreases ACC activity (Corton et al., 1994; Corton et al., 1995; Sullivan et al., 1994; Henin et al., 1995). In 7-day old rabbit hearts we also observed an increase in AMPK activity and a decrease in ACC activity following AICAR administration. This was also accompanied by a significant increase in the rate of fatty acid oxidation, providing further evidence for a role of AMPK in regulating fatty acid oxidation in the newborn heart.

Since AMPK from both 1-day and 7-day old rabbits could be inhibited by insulin, it appears that insulin control of AMPK persists following birth. It is therefore conceivable that the dramatic decrease in insulin levels following birth results in the removal of a physiological inhibitor of AMPK activity (i.e. insulin). This would be expected to increase AMPK activity *in vivo.* As a result, we suggest that changes in insulin levels are responsible for the increase in AMPK activity and the decrease in ACC activity following birth.

Our data also show that AMPK in the newborn heart can be rapidly activated or inhibited, which probably involves a change in the phosphorylated status of AMPK (see Table 4-1 and Figure 4-6). Phosphorylation of AMPK by an upstream AMPK kinase (AMPKK) will activate AMPK, while dephosphorylation of AMPK by phosphatases (particularly protein phosphatase 2A and 2C) will decrease AMPK activity (Hardie et al., 1992). Our results are therefore in agreement with previous findings by Hardie and colleagues (Davies et al., 1995; Hardie et al., 1992). In rat liver, AMP increases AMPK activity by either direct allosteric activation of AMPK, by direct activation of AMPKK, or by facilitating AMPKK phosphorylation of AMPK, or by inhibiting dephosphorylation of AMPK (Davies et al., 1995; Hawley et al., 1995). Our data also provide indirect evidence that cardiac AMPK is also under phosphorylation control and that insulin decreases AMPK activity. While inhibition of AMPK by insulin has been demonstrated in rat liver homogenates and isolated hepatocytes (Hardie, 1992; Witters and Kemp, 1992), the mechanism by which insulin inhibits AMPK in newborn heart remain to be determined.

Altogether, results from this study demonstrate that AMPK is upregulated in the immediate newborn period and may be directly involved in the regulation of

ACC and fatty acid oxidation in newborn hearts. It appears that the rapid decline in insulin levels following birth, results in an increased AMPK activity with the resultant increase in phosphorylation and inhibition of ACC activity. This may lead to low levels of malonyl CoA and increased CPT 1 activity. The overall result is an increase in translocation of activated fatty acids into mitochondria for ß-oxidation.

Table 4-1

Effects of *in vitro* incubation of 6% PEG 8000 extracts (obtained from 1 and 7-day old rabbit hearts perfused in the presence or absence of insulin) with ATP/Mg^{2+} on AMPK activity.

Incubation Condition	AMPK activity (*pmol / min /mg protein*)	
	-AMP	*+ 200 μM AMP*
Control		
1-day old		
- No pre-incubation	247 ± 20	585 ± 75
- Control ,,	149 ± 21	325 ± 21
-ATP/Mg^{2+}	415 ± 34*	1396 ± 51*
7-day old		
- No pre-incubation	467 ± 50	909 ± 60
- Control ,,	126 ± 10	330 ± 49
-ATP/Mg^{2+}	234 ± 24*	445 ± 31
Insulin-treated		
1-day old		
- No pre-incubation	160 ± 20	230 ± 28
- Control ,,	149 ± 10	271 ± 71
-ATP/Mg^{2+}	343 ± 18*	613 ± 56*
7-day old		
- No pre-incubation	241 ± 19	353 ± 25
- Control ,,	147 ± 24	201 ± 12
-ATP/Mg^{2+}	254 ± 21*	438 ± 34*

Values are mean ± SEM; n=6 in each group; * significantly different from control.

Table 4-2

Effects of 5-amino 4-imidazolecarboxamide riboside (AICAR) on AMPK activity, ACC activity and palmitate oxidation rates in 7-day old rabbit hearts.

	Control	AICAR (200 µM)
AMPK Activity (pmol / min / mg protein)	838 ± 42	1175 ± 20*
ACC Activity (nmol / min / mg protein)	2.43 ± 0.10	1.88 ± 0.04*
Palmitate Oxidation (nmol /min / g dry wt)	23.7 ± 1.87	49.5 ± 12.0*

* Significantly different from control.

Values are the mean ± SEM of results from 6 control and 6 AICAR-treated hearts.

Figure 4-1

AMPK activity in unperfused 1-day and 7-day old hearts. AMPK activity was determined in the presence or absence of 200 µM AMP as in Methods.

Values are the mean ± S.E.M. of results from 6 hearts in each group.

* significantly different from 0 AMP by Student's 't' test.

† significantly different from comparable 1-day old heart by analysis of variance followed by Neuman-Keul's test.

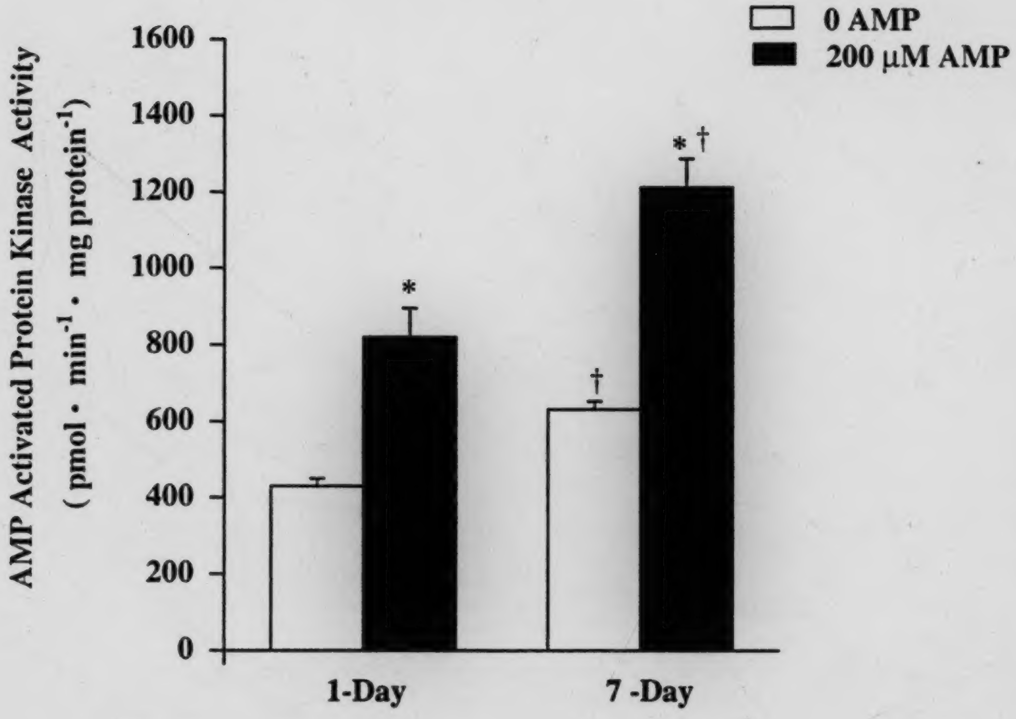

0 AMP
200 μM AMP
AMP Activated Protein Kinase Activity
(pmol • min-1 • mg protein-1)
1600
1400
1200
1000
800
600
400
200
0
*
* †
†
1-Day
7 -Day

Figure 4-2

Representative immunoblot analysis of AMPK in unperfused 1-day and 7-day old hearts using polyclonal anti-AMPK catalytic subunit antibody as described in Methods. AMPK is upregulated in 7-day old heart.

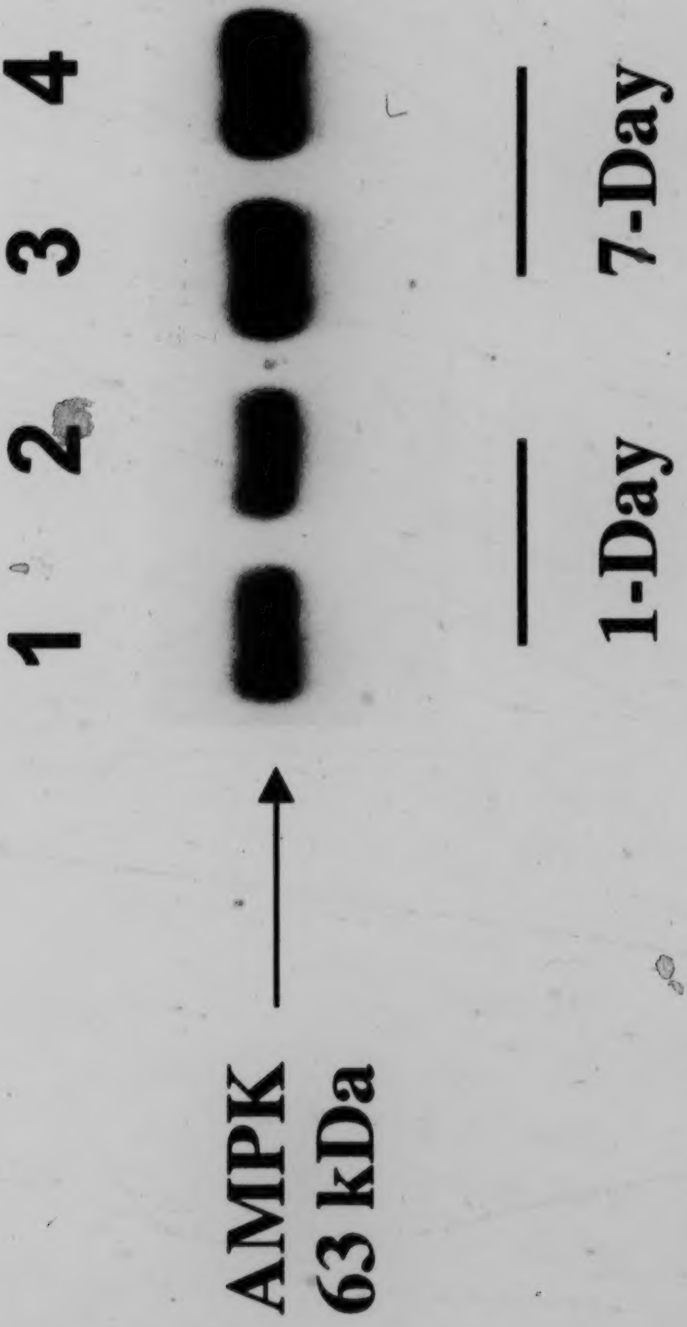

AMPK
63 kDa
1
2
3
4
1-Day
7-Day

Figure 4-3

Palmitate oxidation rate in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 μU/ml). Palmitate oxidation was determined as in Methods.

Values are the mean ± S.E.M. of results from 8 hearts in each group.

* significantly different from controls by unpaired Student's 't' test.

† significantly different from comparable 1-day old heart by analysis of variance followed by Neuman-Keul's test.

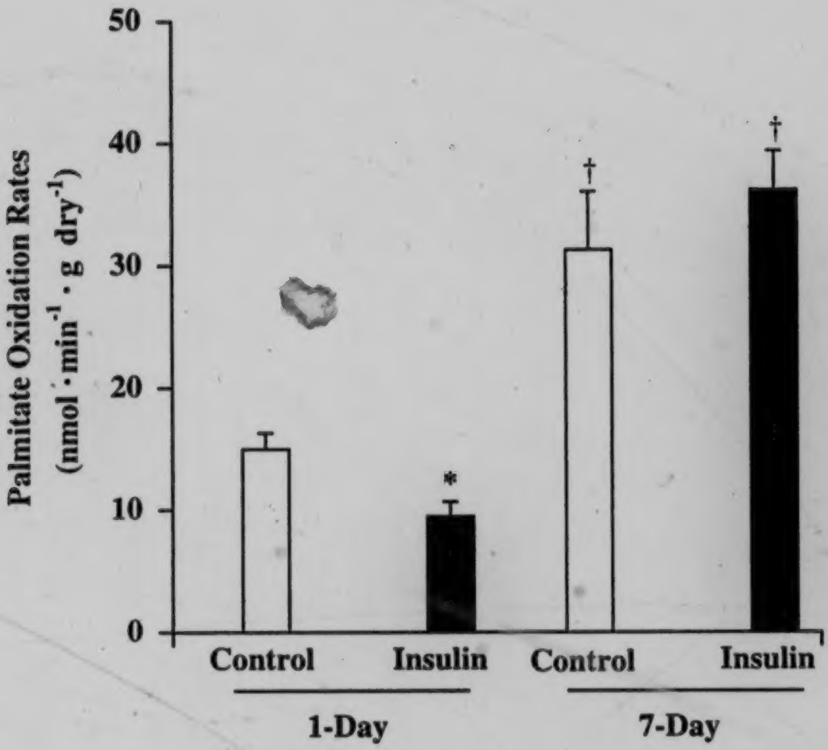

Palmitate Oxidation Rates
(nmol · min-1 · g dry-1)
50
40
30
20
10
0
*
†
†
Control
Insulin
Control
Insulin
1-Day
7-Day

Figure 4-4

ACC activity in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 μU/ml). 6% PEG 8000 extracts were used to determine ACC activity by $^{14}CO_2$ fixation assay as in Methods.

Values are the mean ± S.E.M. of results from 8 hearts in each group.

* significantly different from controls by unpaired Student's 't' test.

† significantly different from comparable 1-day old heart by analysis of variance followed by Neuman-Keul's test.

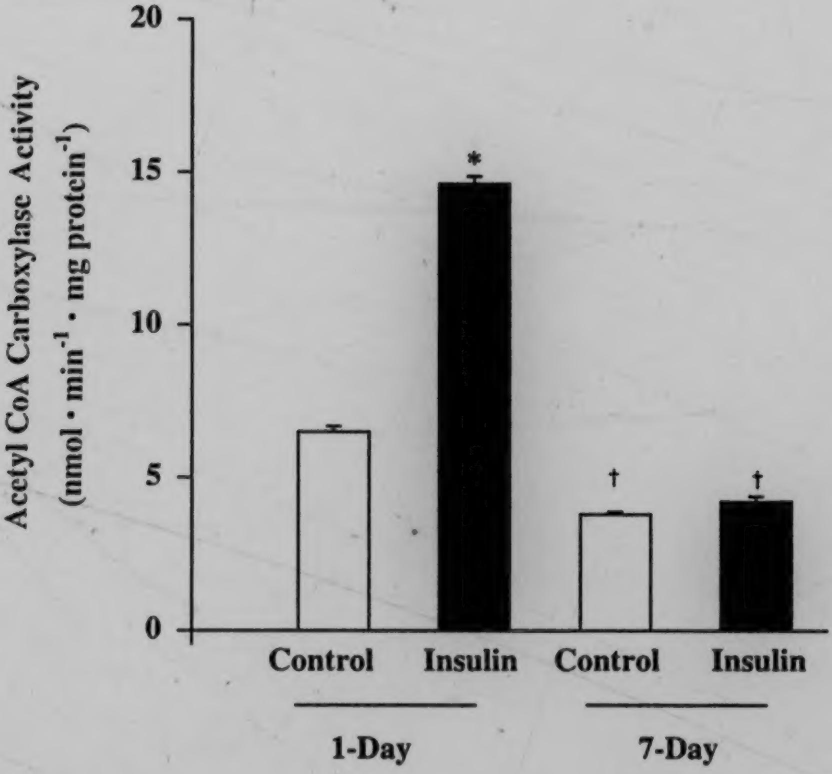

Acetyl CoA Carboxylase Activity
(nmol · min⁻¹ · mg protein⁻¹)
20
15
10
5
0
*
†
†
Control
Insulin
Control
Insulin
1-Day
7-Day

Figure 4-5

AMPK activity in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 μU/ml). AMPK activity was determined in the presence or absence of 200 μM AMP as in Methods.

Values are the mean ± S.E.M. of determinations from 8 hearts in each group.

* significantly different from 0 AMP by unpaired Student's 't' test

† significantly different from comparable 1-day old heart by analysis of variance followed by Neuman-Keul's test.

a significantly different from controls in each group by unpaired Student's 't' test.

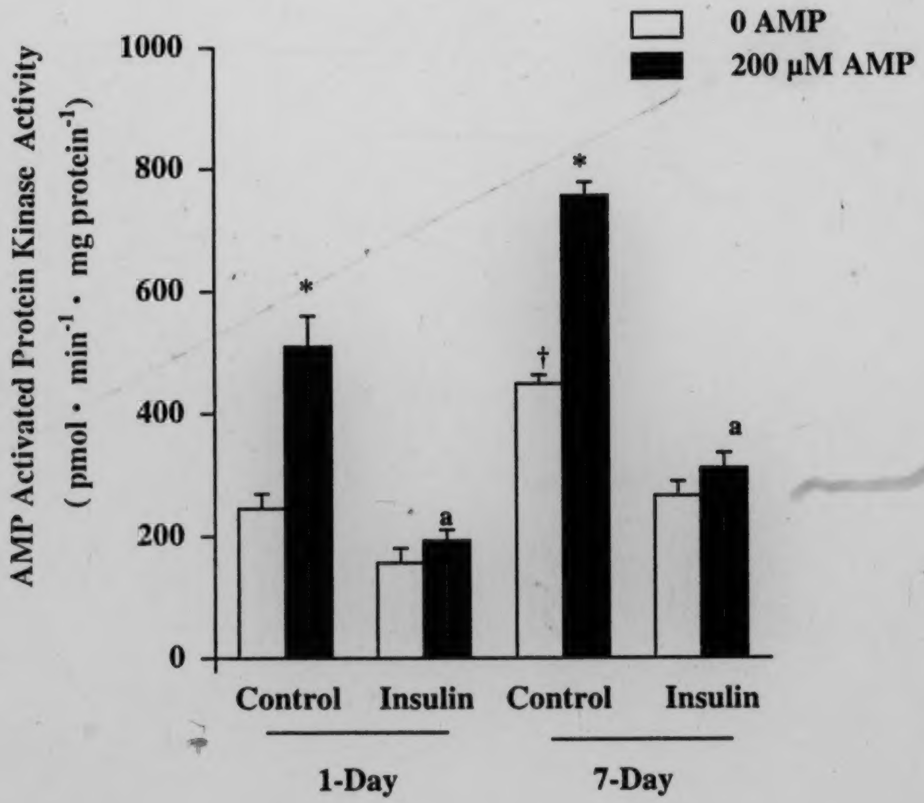

0 AMP
200 µM AMP
AMP Activated Protein Kinase Activity
(pmol • min^{-1} • mg protein^{-1})
1000
800
600
400
200
0
*
*
†
a
a
Control
Insulin
Control
Insulin
1-Day
7-Day

Figure 4-6

AMPK activity in 1-day and 7-day old hearts perfused in the presence or absence of insulin (100 µU/ml) following pre-incubation with PP2A. 6% PEG 8000 extracts were pre-incubated for 30 min with PP2A as in Methods. AMPK activity was then determined in the presence or absence of 200 µM AMP as in Methods.

Values are the mean ± S.E.M. of results from 8 hearts in each group.

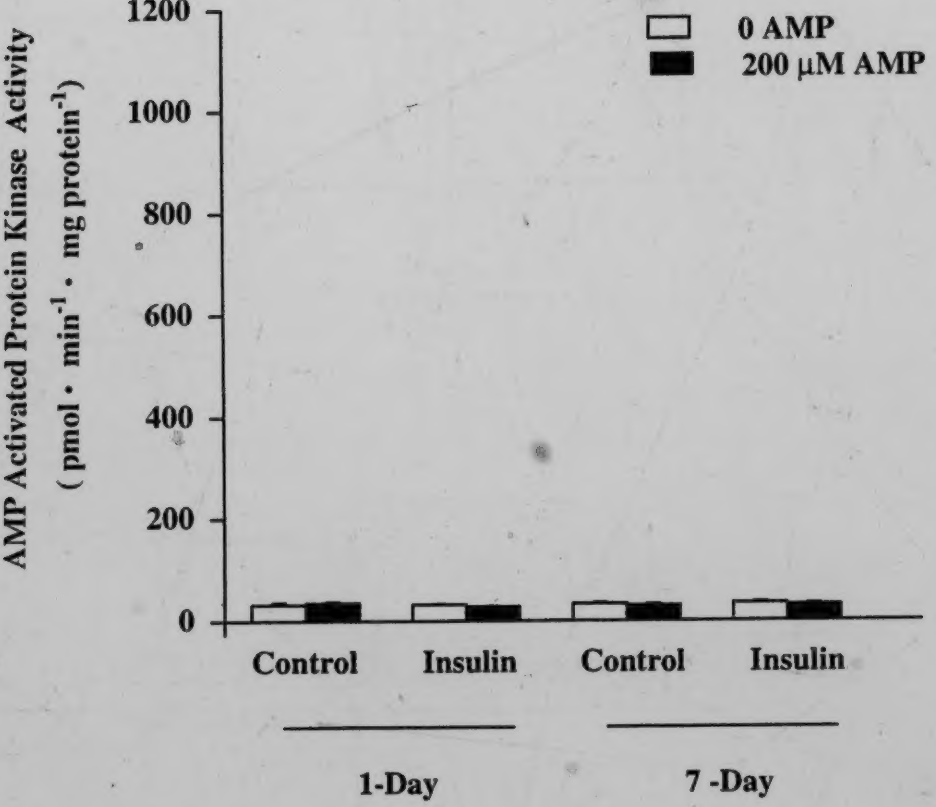
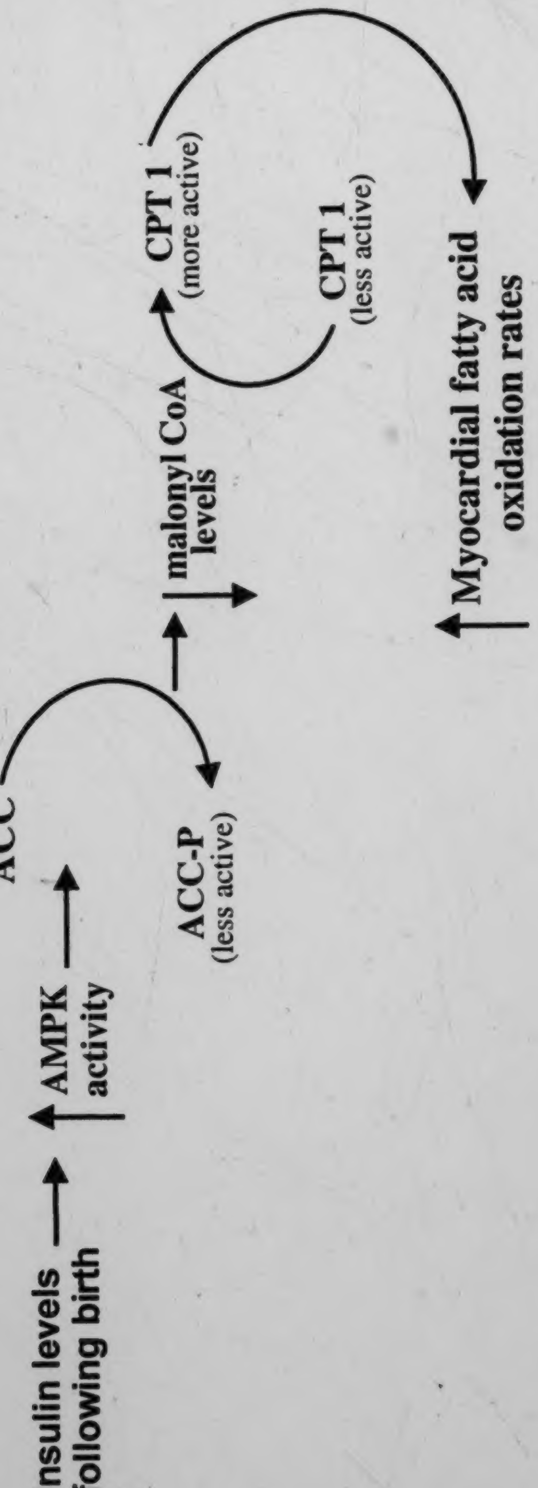

AMP Activated Protein Kinase Activity
(pmol • min^{-1} • mg protein^{-1})
1200
1000
800
600
400
200
0
0 AMP
200 μM AMP
Control
Insulin
Control
Insulin
1-Day
7 -Day

Figure 4-7

Proposed scheme showing the mechanism by which decreased circulating insulin levels following birth may result in an increase in fatty acid oxidation.

The decrease in circulating insulin levels that occur following birth results in an activation of AMPK. This results in a phosphorylation and inhibition of ACC with a resultant decrease in malonyl CoA levels. This relieves the inhibition of malonyl CoA on CPT 1, the rate-limiting enzyme involved in fatty acid transport into mitochondria. The result is an increase in fatty acid oxidation that provides a greater proportion of myocardial ATP demand.


↓ Insulin levels following birth
↑ AMPK activity
ACC
ACC-P (less active)
↓ malonyl CoA levels
CPT 1 (less active)
CPT 1 (more active)
↑ Myocardial fatty acid oxidation rates

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Chapter 5

Evidence that 5'AMP-activated protein kinase activity regulates myocardial fatty acid oxidation rates in newborn rabbit hearts

Introduction

We demonstrated previously that upregulation of AMP-activated protein kinase (AMPK) is responsible for the decreased acetyl CoA carboxylase (ACC) activity and the increased myocardial fatty acid oxidation in 7-day compared to 1-day old heart. Although increased AMPK activity was associated with decreased ACC activity in 7-day old heart, direct evidence that supports AMPK regulation of ACC in newborn heart has yet to be provided. AMPK can phosphorylate and inactivate rat heart ACC in an *in-vitro* assay (Dyck et al., 1998). Whether stimulation or inhibition of AMPK, respectively, results in decreased or increased ACC activity in newborn heart remains unknown. Furthermore, it has yet to be determined if direct stimulation or inhibition of AMPK, respectively, results in increased or decreased fatty acid oxidation secondary to altered ACC activity. We hypothesize that agents which activate AMPK will decrease ACC and increase fatty acid oxidation, while agents which inhibit AMPK will increase ACC activity and decrease fatty acid oxidation.

Pharmacologically, AMPK has been shown in liver and adipose tissue to be stimulated by 5-amino-4-imidazolecarboxamide ribotide (AICAR). AICAR is a cell permeable nucleoside, which can be taken up by cells and phosphorylated by adenylate kinase to form 5-aminoimidazole-4-carboxamide riboside monophosphate (ZMP) (Corton et al., 1995; Sullivan et al., 1994). ZMP then acts as an analogue of AMP to activate AMPK, both allosterically and by facilitating the phosphorylation and activation of AMPK by an AMP-activated protein kinase kinase (AMPKK) (Weekes et al., 1994). In liver and adipose tissue AICAR not only activates AMPK, but also inhibits ACC activity and decreases fatty acid biosynthesis. Recently, Henin et al. (1996) demonstrated in hepatocytes that AMPK could be inhibited with 5-iodotubercidin and 2'deoxyadenosine. Whether or not these agents inhibit AMPK in the newborn heart remains to be investigated.

The purpose of this study was to determine whether AICAR or iodotubercidin or 2'deoxyadenosine can modulate AMPK activity, ACC activity, levels of malonyl CoA and fatty acid oxidation rate in newborn hearts. This was accomplished in 7-day old isolated working rabbit hearts in which AMPK activity and fatty acid oxidation were directly measured. Our data provide evidence that in the newborn heart AMPK is an important regulator of myocardial fatty acid oxidation.

Experimental Procedures

Materials:

AICAR and 2'deoxyadenosine were obtained from the Sigma Chemical Co. Iodotubercidin was obtained from Research Biochemicals International (RBI) and from Drs. Alan Patterson and Wendy Gati, University of Alberta. Anti AMPK catalytic subunit antibody was a gift from Dr. D.G. Hardie (Dundee, Scotland). All other chemicals and materials were obtained as described in Methods

Heart Perfusions:

Hearts from 7-day old New Zealand white rabbits (either sex) were used in this study. Hearts were perfused in the working mode as described in the "Methods". All hearts were perfused with Krebs'-Henseleit solution containing 11 mM glucose and 0.4 mM [1-^{14}C] palmitate prebound to 3% bovine serum albumin. Hearts were subjected to a 40 min aerobic perfusion at a 7.5 mm Hg left atrial preload and 30 mm Hg aortic afterload, either in the presence or absence of

AICAR (200 μM), iodotubercidin (50 μM) or 2'deoxyadenosine (300 μM). Steady state palmitate oxidation rates were measured over a 40 min aerobic period as described in the Methods. At the end of the experiments ventricles were quickly frozen using Wollenberger tongs pre-cooled to the temperature of liquid nitrogen and stored at – 80 °C until ready for analysis.

Tissue preparations:

Frozen ventricular tissues were used to prepare PEG 8000 extracts as described in the "Methods".

Measurement of AMP-activated protein kinase and acetyl CoA carboxylase activity:

AMPK and ACC activity in 6% PEG 8000 extracts obtained from ventricular tissues were measured as described in the "Methods".

Western blot analysis of ACC and AMPK:

To determine if changes in ACC or AMPK activity by iodotubercidin resulted from changes in ACC or AMPK protein abundance during the perfusion period, samples of 6% PEG 8000 extracts obtained from control and iodotubercidin perfused hearts were subjected to a 5% and 9% SDS-PAGE for ACC and AMPK proteins, respectively, as described in the "Methods". Following gel electrophoresis fractionated proteins were transferred to nitrocellulose membrane. AMPK protein was detected using an anti-AMPK α2 catalytic subunit antibody, kindly provided by Dr. Graham Hardie (Dundee, Scotland). This was followed by chemiluminescent detection as described in the "Methods". ACC protein content was determined by following procedures described in the "Methods".

Determination of CoA esters and nucleotide levels

Approximately 400 mg portion of the powdered frozen ventricular tissue was used for a 6% perchloric acid (PCA) extraction, as in the "Methods". CoA esters and nucleotide levels were then determined as described in the "Methods".

Linear regression and correlation analysis:

Results from the AMPK, ACC and fatty acid oxidation experiments were subjected to linear regression and correlation analysis using an Instat$^{@}$ statistical package for regression analysis correlation coefficient determinations.

Statistical analysis:

Data are expressed as the mean ± standard error of the mean. Comparisons were performed using the unpaired Student's t-test where appropriate. When three or more group means were compared, analysis of variance followed by Neuman-Keul's test was used to determine statistical significance between groups. Statistical level of significance was set at $p < 0.05$.

Results

Mechanical function in 7-day old rabbit hearts perfused with AICAR or iodotubercidin.

Shown in Table 5-1 is mechanical function measured in control, AICAR-treated and iodotubercidin-treated hearts. Heart function was stable throughout the 40 min aerobic perfusion period and was not significantly different between any experimental group. Although iodotubercidin resulted in a small non-significant decrease in heart rate and cardiac output, this was accompanied by an increase in peak systolic pressure, such that HRxPSP and cardiac work were similar between groups. It is however interesting to note that 2'deoxyadenosine significantly increased cardiac work (Table 5-1).

Fatty acid oxidation rates in AICAR and iodotubercidin perfused hearts:

Steady state rates of palmitate oxidation in control, AICAR and iodotubercidin-treated hearts are shown in Figure 5-1. Rates of palmitate oxidation seen in the control group are comparable to that previously observed in 7-day old hearts perfused under identical conditions (Itoi and Lopaschuk, 1993; Itoi and Lopaschuk, 1996). If 200 μM AICAR was present in the perfusate, a significant increase in rates of fatty acid oxidation were observed compared to control hearts. In contrast, addition of 50 μM iodotubercidin to the perfusate resulted in a significant decrease in rates of palmitate oxidation.

AMPK and ACC activity in AICAR and iodotubercidin perfused hearts

AMPK activity measured in frozen ventricular tissues at the end of 40 min perfusion period is shown in Figure 5-2. In 7-day old control hearts, AMPK activity was detected in 6% PEG 8000 extracts incubated in the absence of AMP. Addition of 200 μM AMP resulted in a further activation of AMPK activity. In AICAR-treated hearts AMPK activity was significantly stimulated compared to controls both in the absence or presence of exogenously added AMP. In contrast, AMPK activity in iodotubercidin-treated hearts decreased significantly compared to controls regardless of whether AMP was absent or present in the assay medium.

ACC activity in control, AICAR-treated and iodotubercidin-treated hearts is shown in Figure 5-3. AICAR-treatment of hearts resulted in a significant decrease in ACC activity, which is consistent with an increased phosphorylation and inhibition of ACC by AMPK. In contrast, iodotubercidin-treatment resulted in a significant increase in ACC activity compared to controls.

CoA esters and nucleotide levels in AICAR and iodotubercidin perfused hearts:

Malonyl CoA levels in hearts frozen at the end of the perfusion period are shown in Figure 5-4. Malonyl CoA levels in control hearts were similar to values previously reported in 7-day old rabbit hearts perfused under identical conditions (Lopaschuk et al., 1994). In AICAR-treated hearts, a significant decrease in malonyl CoA levels was observed. In contrast, an increase in malonyl CoA levels was observed in iodotubercidin-treated hearts compared to controls.

Levels of acetyl CoA in control, AICAR-treated and ITC-treated hearts were 13.65 ± 0.28, 7.6 ± 0.9, and 5.2 ± 0.35 nmol/g dry wt, respectively. The decreased acetyl CoA levels in iodotubercidin-treated hearts compared to control hearts may be indicative of lower rates of palmitate oxidation and a subsequent decrease in acetyl CoA derived from β-oxidation. Since ACC activity increased in iodotubercidin-treated hearts, results from this study implies that changes in ACC activity and malonyl CoA levels result from direct phosphorylation control of ACC, as opposed to changes in acetyl CoA supply for the two step carboxylation of acetyl CoA by ACC.

Shown in Table 5-2 are the nucleotide levels in control, AICAR and iodotubercidin treated hearts. Neither AICAR or iodotubercidin significantly altered AMP or ATP levels. In addition, the AMP /ATP ratio in AICAR or iodotubercidin treated hearts was not significantly different from controls.

AMPK and ACC protein in AICAR and iodotubercidin perfused hearts:

To determine whether or not changes in AMPK and ACC activity resulted from AICAR or iodotubercidin-induced alterations in AMPK or ACC protein levels, western blot analysis of 6% PEG 8000 extracts obtained from hearts treated with these agents was carried out. As can be seen in Figure 5-5(A), iodotubercidin did not alter the amount of the 63 kDa catalytic subunit of AMPK detected by western blot analysis, compared to control. Iodotubercidin-treatment also did not alter the amount of two isoforms of the ACC 280 and 265 kDa detected by streptavidin conjugated horseradish peroxidase (Figure 5-5B). These results suggest that any change in ACC activity is due to altered allosteric modification of ACC, possibly by changes in phosphate incorporation into ACC.

AMPK, ACC activity and fatty acid oxidation in 2'deoxyadenosine treated hearts:

To further demonstrate that alterations in AMPK activity result in changes in myocardial fatty acid oxidation rates, and to address the specificity of the AMPK inhibitor iodotubercidin, another different series of hearts was perfused with 300 μM 2'deoxyadenosine, an inhibitor of AMPK. In this series of hearts, we tested the hypothesis that 2'deoxyadenosine by inhibiting AMPK activity will decrease rates of palmitate oxidation. Shown in Table 5-3 is the effect of 2'deoxyadenosine on AMPK, ACC and palmitate oxidation. 2'deoxyadenosine significantly decreased AMPK activity and increased ACC activity compared to controls. Although chemically different from iodotubercidin, the effects of 2'deoxyadenosine in inhibiting AMPK activity are comparable to those of iodotubercidin (Figure 5-2).

Cardiac work is an important determinant of the rate of fatty acid oxidation in the heart. Since 2'deoxyadenosine increased cardiac work, palmitate oxidation was normalized for cardiac work performed by the heart. As shown in Table 5-3, 2'deoxyadenosine also significantly decreased rates of palmitate oxidation during the 40 min aerobic perfusion period compared to controls (Table 5-3). It would appear that inhibition of AMPK activity has the potential to relieve the phosphorylation and inactivation of ACC, such that ACC remains in its active dephosphorylated state. These data provide additional support for the concept that inhibition of AMPK results in activation of ACC and an inhibition of fatty acid oxidation.

Correlation between AMPK activity, ACC activity, and fatty acid oxidation in 7-day old hearts

A plot of AMPK activity versus ACC activity showed a significant negative correlation (Figure 5-6). The correlation between AMPK activity and rate of fatty acid oxidation measured in our experimental groups is shown in Figure 5-7. A significant positive correlation was observed. Finally, a significant negative correlation was also observed between ACC activity and rates of fatty acid oxidation (Figure 5-8).

Discussion:

The results from our studies provide evidence that AMPK regulates fatty acid oxidation in the newborn heart. Previous studies in the post-ischemic heart (Kudo et al., 1995; Kudo et al., 1996) have shown that changes in AMPK activity are accompanied by parallel changes in fatty acid oxidation, although a cause and effect relationship was not provided. Using a pharmacological approach we show that stimulation of AMPK results in a decreased ACC activity and malonyl CoA levels, and an increase in fatty acid oxidation rate. In contrast, when AMPK was inhibited using iodotubercidin, an increase in ACC activity was observed, which was accompanied by an increase in levels of malonyl CoA and a decrease in rates of fatty acid oxidation. Based on these results, we suggest that a key role for the high AMPK protein abundance and activity seen in the heart following birth is to regulate fatty acid oxidation.

Pharmacological modification of AMPK activity

Previous studies in hepatocytes and adipose tissues have shown that AICAR, (a cell permeable analogue of adenosine which is rapidly taken into the cells possibly via an adenosine transport system) is rapidly converted to ZMP (Corton et al., 1995). The increase in ZMP results in an increase in AMPK activity and a parallel decrease in ACC activity (Corton et al., 1995; Sullivan et al., 1994). Although a role for ACC in regulating fatty acid oxidation in the heart and skeletal muscle has now been firmly established (Saddik et al., 1993; Lopaschuk and Gamble, 1994), no previous studies have looked at the relationship between AICAR, AMPK, ACC and rates of fatty acid oxidation in newborn heart muscle. As shown in this study, a good correlation between AMPK activity, ACC activity and fatty acid oxidation was observed, confirming the importance of AMPK and ACC as regulators of fatty acid oxidation in the heart. The results from this study is therefore in agreement with findings by Merrill et al. (1997), which demonstrated that AICAR increases both AMPK activity and fatty acid oxidation in rat skeletal muscle.

Iodotubercidin and 2'deoxyadenosine was recently shown by Henin et al. (1996) to be an effective inhibitor of isolated liver AMPK. Although iodotubercidin is an AMP analogue, studies by Massilon et al. (1994) suggest that iodotubercidin inhibits adenosine kinase and phosphorylase kinase, and may be a general protein kinase inhibitor. However, this suggestion is at variance with results of Fluckiger-Isler and colleagues (Fluckiger-Isler et al., 1996), who found that iodotubercidin may not be a general protein kinase inhibitor. In our hands, iodotubercidin inhibits AMPK activity and lowers rate of fatty acid oxidation. Regardless of specificity, we demonstrate that iodotubercidin inhibits AMPK activity and decreases rate of fatty acid oxidation in newborn hearts. These effects of iodotubercidin are unlikely to be due to an inhibition of adenosine kinase or phosphorylase kinase (Dr. Van den Berghe personal communication). Whereas it is possible that iodotubercidin may inhibit fatty acid oxidation independent of its effect on AMPK, neither adenosine kinase nor phosphorylase kinase has been shown to be directly involved in the metabolism of fatty acids. Of interest, is that another pharmacological agent, 2'deoxyadenosine, also inhibits AMPK activity and significantly decreases palmitate oxidation rates in newborn rabbit hearts in a fashion similar to that obtained by iodotubercidin.

The striking positive correlation between inhibition of AMPK and the inhibition of fatty acid oxidation seen in our study strongly suggest that the effects of iodotubercidin are occurring secondary to a direct inhibition of AMPK. Iodotubercidin being a purine analogue, may be acting as a competitive antagonist at the AMP binding site on AMPK, but this remains speculative. Certainly, iodotubercidin has no effect on AMPK abundance in the perfused newborn hearts (Figure 5-5A). Decreased AMPK activity by iodotubercidin may therefore not result from altered protein stability or degradation. Another possible explanation for the mechanism of iodotubercidin action is a possible inhibition of an upstream AMPK kinase (AMPKK, known to phosphorylate and activate AMPK). AMP can both directly stimulate AMPKK and promote AMPKK phosphorylation and activation of AMPK. Unfortunately, iodotubercidin has no significant effect on either AMP levels or AMP/ATP ratio in our hands (see Table 5-2). It is likely that iodotubercidin prevents the phosphorylation and activation of AMPK by AMPKK, a possibility supported by the observation that AMPK activity was still very low in iodotubercidin-treated hearts even in the presence of added AMP (Figure 5-2). Certainly, the mechanism by which iodotubercidin inhibits AMPK activity to alter myocardial fatty acid oxidation merits further investigation.

A proposed scheme showing how AICAR and iodotubercidin modify fatty acid oxidation in the heart is shown in Figure 5-9. By stimulating or inhibiting AMPK, phosphorylation and inactivation of ACC (and subsequent malonyl CoA production) is either increased or decreased, respectively. Since malonyl CoA is a potent inhibitor of CPT 1, the rate-limiting enzyme involved in mitochondrial fatty acid uptake (McGarry et al., 1983), the AICAR-induced decrease in malonyl CoA levels results in an increase in rates of fatty acid oxidation, while iodotubercidin-induced increase in malonyl CoA inhibits rates of fatty acid oxidation.

In summary, we show that AICAR or iodotubercidin increases and decreases, respectively, AMPK activity. This results in a decrease or increase in ACC activity and malonyl CoA levels, respectively. The ultimate result is an increase or decrease fatty acid oxidation in newborn hearts, respectively, by AICAR or iodotubercidin. 2'Deoxyadenosine another AMPK inhibitor, decreases fatty acid oxidation secondary to an inhibition of AMPK activity. We provide evidence that myocardial AMPK is an important regulator of fatty acid oxidation in newborn hearts.

Table 5-1:

Effect of AICAR and iodotubercidin and 2'deoxyadenosine on mechanical function in isolated working hearts from 7-day old rabbits.

Parameter Measured	Control	AICAR	ITC	DeoxyADO
Heart Rate (beats. min^{-1})	220 ± 6	210 ± 9	190 ± 8	200 ± 10
Peak Systolic Pressure (mmHg)	43 ± 2	47 ± 4	49 ± 3	51 ± 2*
Developed Pressure (mmHg)	35 ± 2	34 ± 3	37 ± 2	35 ± 2
HR x PSP (mmHg.beat.min^{-1}.10^{2})	9.46 ± 1.6	9.67 ± 1.9	9.50± 2.0	10.4± 2.0
Cardiac Output (ml.min^{-1})	26 ± 2	23 ± 5	20 ± 3	32 ± 3*
Aortic Flow (ml.min^{-1})	10 ± 3	7 ± 2	6 ± 1	20 ± 3*
Coronary Flow ml.min^{-1})	16 ± 2	15 ± 5	14 ± 3	18 ± 2
Cardiac Work (mmHg.ml.min^{-1}. 10-^{2})	11.08 ± 1.5	10.92 ± 2.2	9.60 ±1.2	16.76 ± 1.8*

Values are the mean ± SEM of 8 control, 5 AICAR, 7 iodotubercidin and 8 2'deoxyadenosine-treated hearts.

* significantly different from control hearts. ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine.

Table 5.2:

Effect of AICAR and iodotubercidin on nucleotide levels in isolated working hearts from 7-day old rabbits.

Perfusion Condition	**ATP** (µmol / g dry wt)	**AMP** (µmol / g dry wt)	**AMP/ATP Ratio**
Control	17.6 ± 1.4	4.8 ± 0.6	0.27 ± 0.08
AICAR	20.6 ± 1.7	6.4 ± 0.9	0.31 ± 0.06
ITC	15.6 ± 0.5	4.5 ± 0.7	0.28 ± 0.03

Values are the mean ± SEM of 8 control, 5 AICAR, 7 iodotubercidin-treated hearts.

Table 5-3:

Effect of 2'deoxyadenosine on AMPK, ACC and palmitate oxidation rates normalized for cardiac work in isolated working hearts from 7-day old rabbits

Parameter Measured	Control	DeoxyADO
AMPK activity (pmol / min / mg protein)	945 ± 85	210 ± 25*
ACC activity (nmol / min / mg protein)	2.16 ± 0.08	3.08 ± 0.05*
Palmitate Oxidation (nmol /g dry / ml /mmHg)	14.2 ± 1.2	8.5 ± 0.9*

Values are the mean ± SEM of 8 control and 8, 2'deoxyadenosine-treated hearts.
* significantly different from control hearts. DeoxyADO = 2'deoxyadenosine.

Figure 5-1:

Palmitate oxidation rates in control, AICAR, and iodotubercidin perfused 7-day old rabbit hearts. Palmitate oxidation rates were determined as described in the Methods.

Values are the mean ± S.E.M. of 8 control, 5 AICAR and 7 ITC treated hearts.

* significantly different from control hearts.

ITC = iodotubercidin.

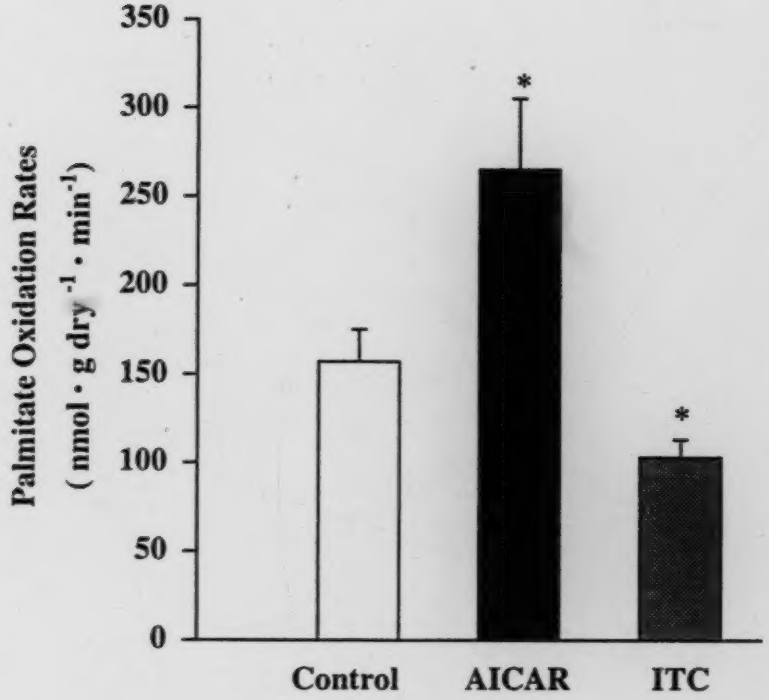

Palmitate Oxidation Rates
(nmol · g dry^{-1} · min^{-1})
350
300
250
200
150
100
50
0
*
*
Control
AICAR
ITC

Figure 5-2:

AMP-activated protein kinase activity in control, AICAR, and iodotubercidin-treated 7-day old rabbit hearts. AMPK activity was measured in the presence or absence of 200 µM AMP, as described in Methods.

Values are the mean ± S.E.M. of 8 control, 5 AICAR and 7 ITC-treated hearts.

* significantly different from 0AMP.

† significantly different from control hearts.

ITC = iodotubercidin.

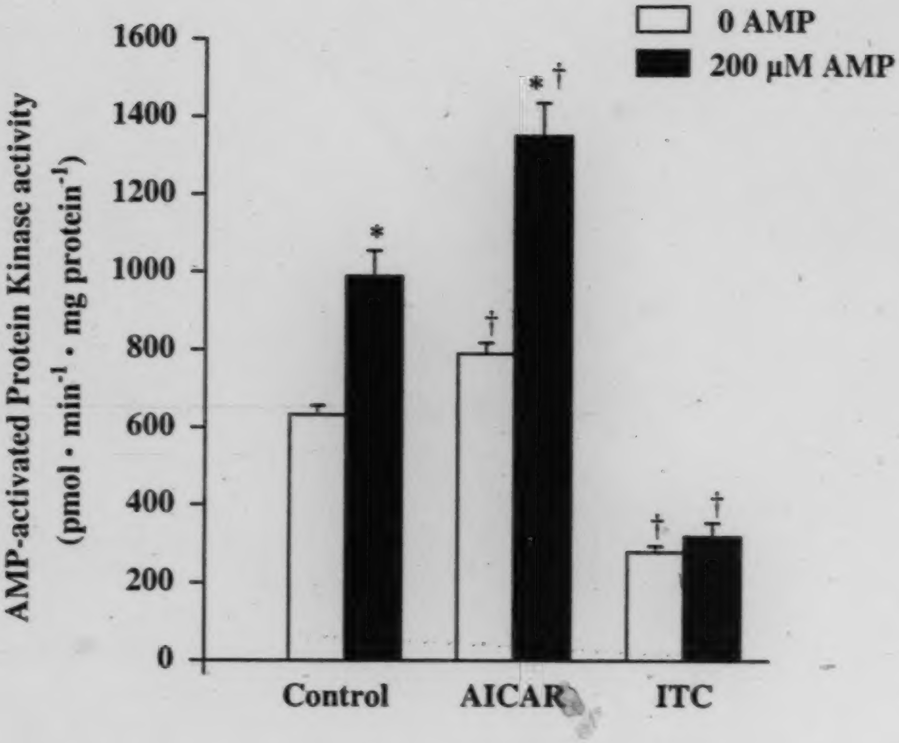

0 AMP
200 μM AMP
AMP-activated Protein Kinase activity
(pmol • min⁻¹ • mg protein⁻¹)
1600
1400
1200
1000
800
600
400
200
0
Control
AICAR
ITC
*
†
* †
†
†

Figure 5-3:

Acetyl CoA carboxylase activity in control, AICAR, and iodotubercidin-treated 7-day old rabbit hearts. ACC activity was measured as described in Methods.

Values are the mean ± S.E.M. of 8 control, 5 AICAR and 7 ITC-treated hearts.

* significantly different from control hearts.

† significantly different from control and AICAR-treated hearts.

ITC = iodotubercidin

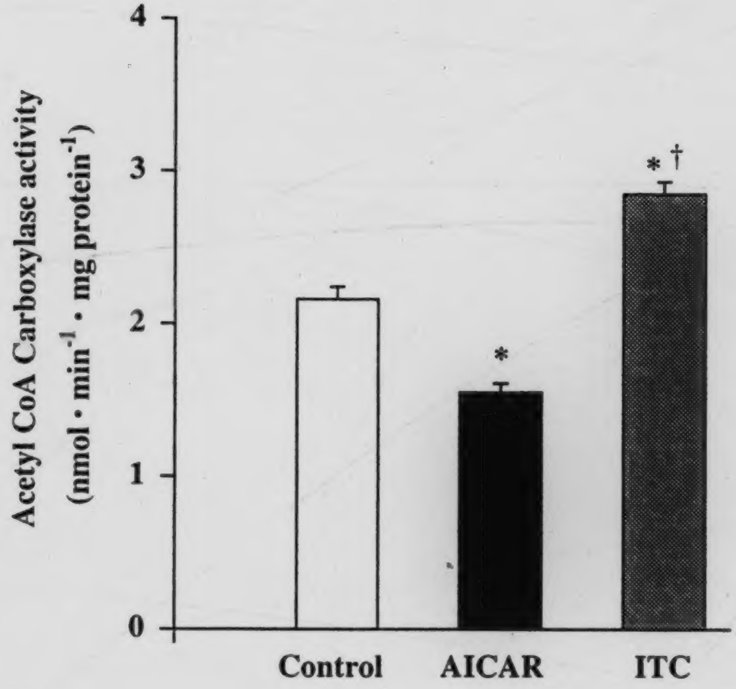

Acetyl CoA Carboxylase activity
(nmol · min^{-1} · mg protein^{-1})
4
3
2
1
0
*
* †
Control
AICAR
ITC

Figure 5-4:

Malonyl CoA levels in control, AICAR, and iodotubercidin-treated 7-day old rabbit hearts. Malonyl CoA levels were measured using an HPLC procedure described in the Methods.

Values are the mean ± S.E.M. of 8 control, 5 AICAR and 7 ITC-treated hearts.

* significantly different from control hearts.

† significantly different from control and AICAR-treated hearts.

ITC = iodotubercidin

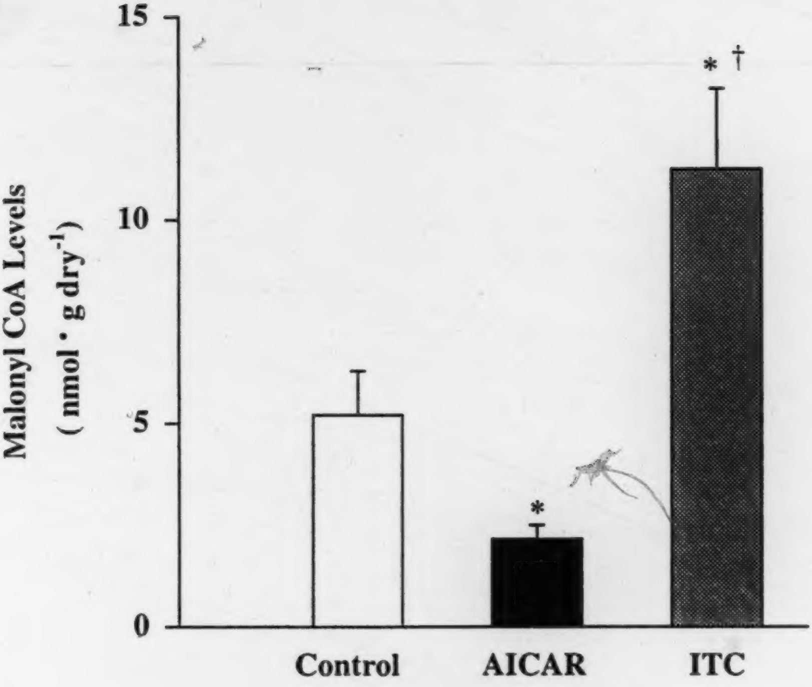

15
10
5
0
Malonyl CoA Levels
(nmol · g dry^{-1})
*
* †
Control
AICAR
ITC

Figure 5-5:

Representative immunoblot analysis showing the effect of iodotubercidin on (A) AMPK catalytic subunit and (B) ACC protein abundance.

Immunoblot analysis of 6% PEG 8000 extracts were performed on 9% and 5% SDS-PAGE for AMPK and ACC respectively as described in Methods.

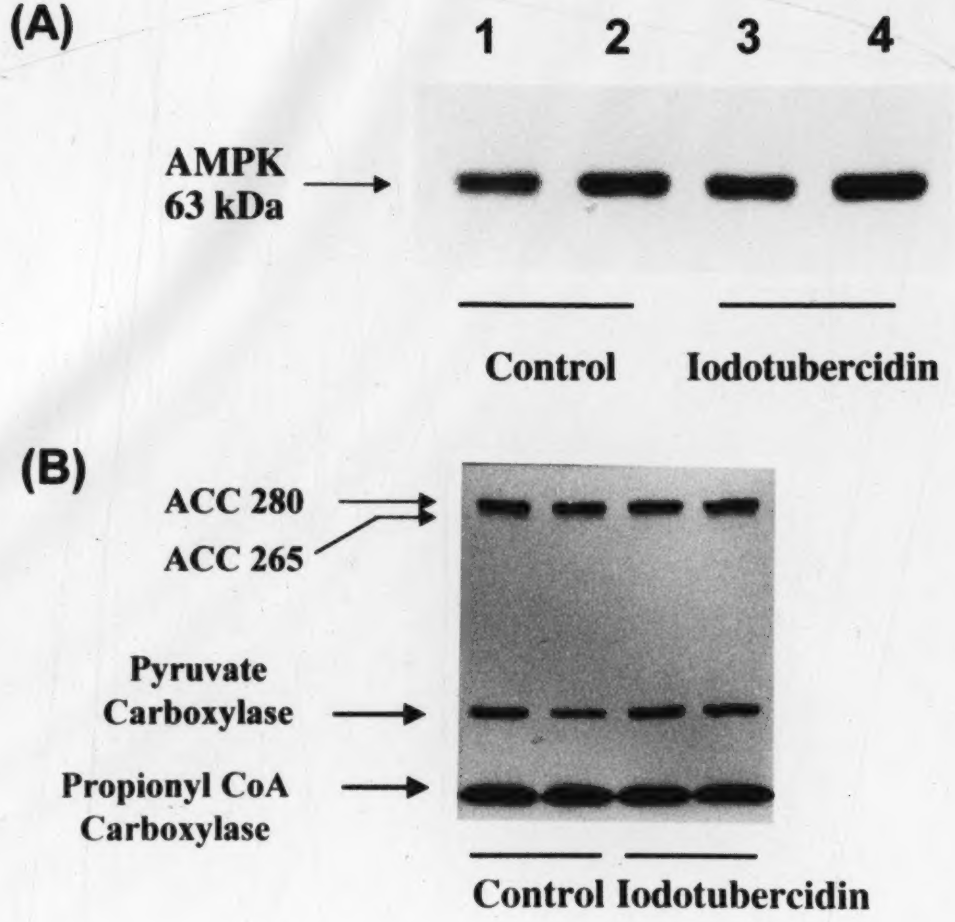

(A)
1
2
3
4
AMPK
63 kDa
Control
Iodotubercidin
(B)
ACC 280
ACC 265
Pyruvate
Carboxylase
Propionyl CoA
Carboxylase
Control
Iodotubercidin

Figure 5-6:

Correlation between AMP-activated protein kinase activity and acetyl CoA carboxylase activity.

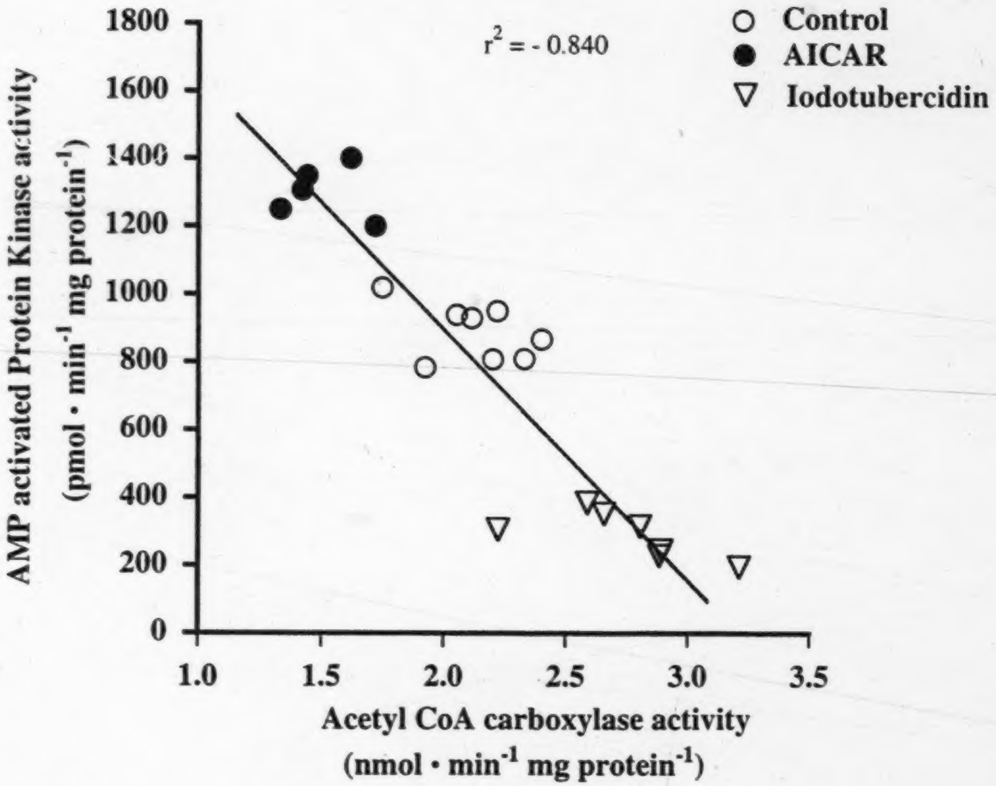

1800
1600
1400
1200
1000
800
600
400
200
0
AMP activated Protein Kinase activity
(pmol · min-1 mg protein-1)
1.0
1.5
2.0
2.5
3.0
3.5
Acetyl CoA carboxylase activity
(nmol · min-1 mg protein-1)
r2 = - 0.840
○ Control
● AICAR
▽ Iodotubercidin

Figure 5-7:

Correlation between AMP-activated protein kinase activity and palmitate oxidation rates.

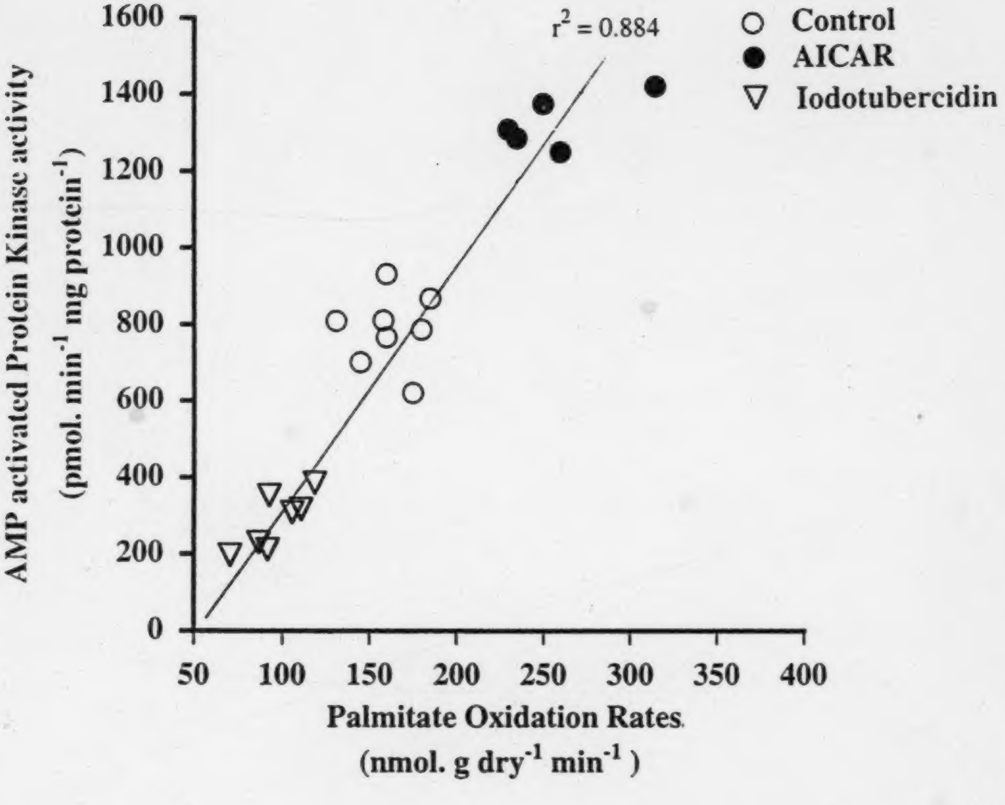
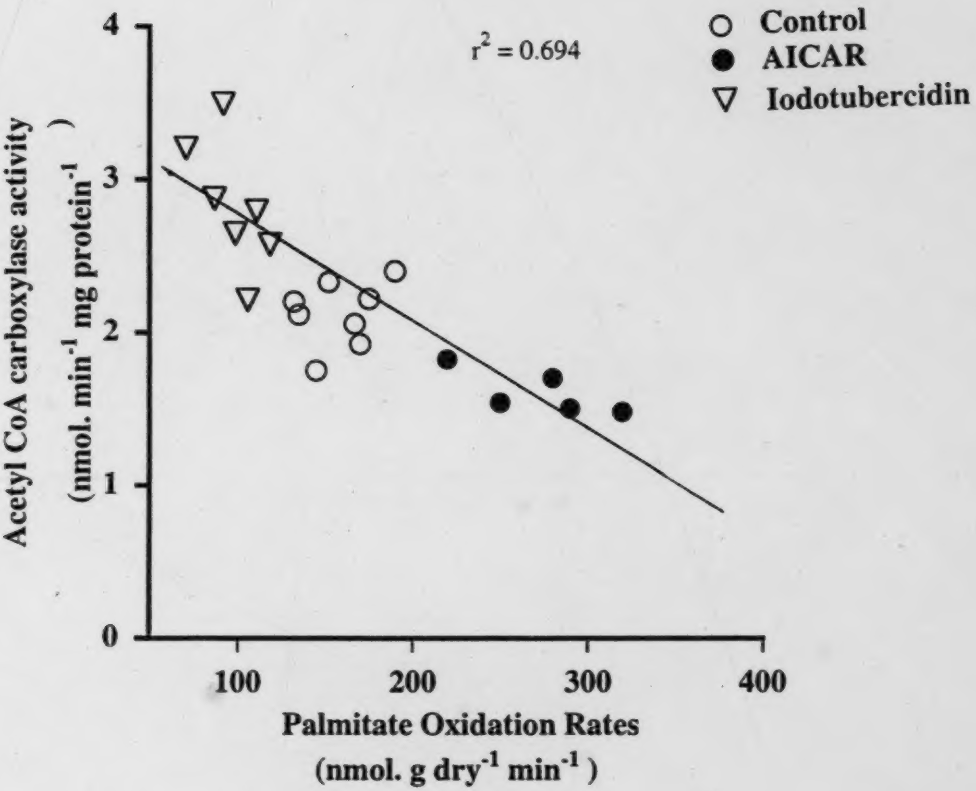

AMP activated Protein Kinase activity
(pmol. min^{-1} mg protein^{-1})
1600
1400
1200
1000
800
600
400
200
0
50
100
150
200
250
300
350
400
Palmitate Oxidation Rates
(nmol. g dry^{-1} min^{-1})
$r^2 = 0.884$
○ Control
● AICAR
▽ Iodotubercidin

Figure 5-8:

Correlation between acetyl CoA carboxylase activity and palmitate oxidation rates.

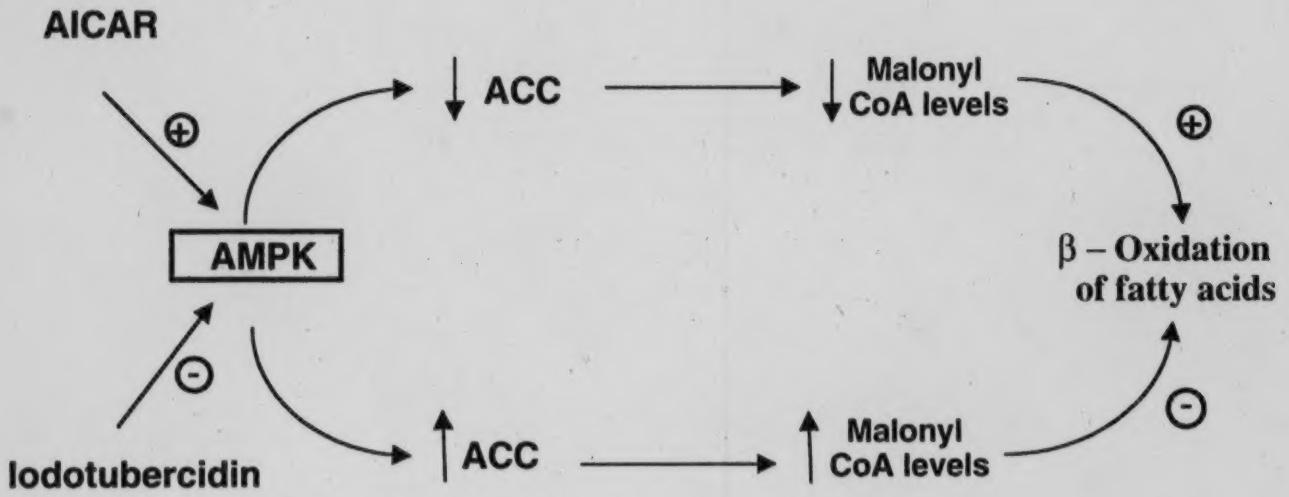


r² = 0.694
○ Control
● AICAR
▽ Iodotubercidin
Acetyl CoA carboxylase activity
(nmol. min⁻¹ mg protein⁻¹)
0
1
2
3
4
100
200
300
400
Palmitate Oxidation Rates
(nmol. g dry⁻¹ min⁻¹)

Figure 5-9:

Proposed scheme showing the mechanism by which AICAR or iodotubercidin alters rate of fatty acid oxidation in the newborn heart.

AICAR by activating AMPK increases the phosphorylation and inactivation of ACC. The resultant decrease in levels of malonyl CoA relieve CPT 1 inhibition, resulting in an increased fatty acid oxidation. Conversely, iodotubercidin, by inhibiting AMPK, results in both an increased ACC activity and malonyl CoA levels resulting in decreased fatty acid oxidation secondary to an inhibition of CPT 1 activity.


AICAR
⊕
AMPK
⊝
Iodotubercidin
↓ ACC
↑ ACC
↓ Malonyl CoA levels
↑ Malonyl CoA levels
⊕
⊝
β – Oxidation of fatty acids

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Kudo, N., Gillespie, J.G., Kung, L., Witters, L.A., Schulz, R., Clanachan, A.S., and Lopaschuk, G.D. (1996) Characterization of 5'AMP-activated protein kinase in the heart and its role in inhibiting acetyl CoA carboxylase during reperfusion following ischemia. *Biochim. Biophys. Acta* **1301,** 67-75.

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CHAPTER 6

5'AMP-activated protein kinase inhibitors improve the recovery of mechanical function following ischemia in newborn rabbit heart

Introduction

In adult rat heart, high rates of fatty acid oxidation during reperfusion of previously ischemic hearts are accompanied with an increase in 5'AMP-activated protein kinase (AMPK), a decrease in acetyl CoA carboxylase (ACC) activity and a depressed functional recovery (Kudo et al., 1995; Kudo et al., 1996). ACC regulates fatty acid oxidation in the heart via its production of malonyl CoA (Saddik et al., 1993), a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1), the rate limiting enzyme for mitochondrial uptake of activated fatty acids (see McGarry and Brown, 1997 for review). Recent studies in our laboratory showed that ACC activity decreased or increased when AMPK was activated or inhibited, respectively, by AICAR or iodotubercidin in newborn hearts. Moreover, an increase or decrease in fatty acid oxidation, respectively, accompanied the activation or inhibition of AMPK (see Chapter 5). To date, the role of AMPK and ACC in the increased fatty acid oxidation and depressed functional recovery following ischemia in newborn hearts has not been determined.

In the clinical setting of myocardial ischemia, increased levels of fatty acid have been reported in infants undergoing cardiac surgery to correct congenital heart defects (Lopaschuk et al., 1994b). High levels of fatty acid are detrimental to recovery of mechanical function in the reperfused-ischemic heart, due in part to inhibitory effects of fatty acid oxidation on glucose oxidation and a resultant imbalance between glycolysis and glucose oxidation (Lopaschuk et al., 1992). Unfortunately, some neonates still need to undergo surgery to correct congenital heart defect. Adequate protection of the heart is therefore important to enhance the recovery of mechanical function post-surgery.

Myocardial ischemia can be regarded as a metabolic disorder, because the primary underlying defect is the inability of the myocardium to synthesize ATP due to an acute shortage of oxygen for oxidative phosphorylation. As a result ATP production is compromised and AMP levels increase due to the extensive hydrolysis of ATP. This results in an increase in the AMP/ATP ratio.

Studies by Moore et al. (1991) and Hawley et al. (1996) showed that increased AMP levels or increased AMP/ATP ratio can result in an activation of AMPK. A recent report by Henin et al. (1996) also demonstrated that AMPK could be inhibited by iodotubercidin or 2'deoxyadenosine in rat hepatocytes. In addition, AMPK activity can be activated by 5-amino-4-imidazolecarboxamide riboside (AICAR) in isolated rat hepatocytes, adipocytes or skeletal muscle (Corton et al., 1995; Sullivan et al., 1994; Young et al., 1996). Whether ischemia results in an activation of AMPK in newborn hearts has yet to be determined. In addition, it is not known whether AMPK inhibitors can lower fatty acid oxidation rates and improve functional recovery in the reperfused-ischemic newborn heart. We hypothesize that AMPK inhibitors improve functional recovery in the reperfused-ischemic newborn heart secondary to a decrease in fatty acid oxidation.

The purpose of this study was to determine if myocardial ischemia results in an activation of AMPK and an acceleration of fatty acid oxidation in the reperfused-ischemic newborn hearts. Moreover, we determined the effect of AMPK inhibitors and activators on fatty acid oxidation and functional recovery in reperfused-ischemic newborn hearts. Our data demonstrate that AMPK inhibitors improve functional recovery of newborn hearts following ischemia.

Experimental Procedures

Materials:

5-Amino-4-imidazolecarboxamide ribotide (AICAR) and 2'deoxyadenosine (DeoxyADO) were obtained from the Sigma Chemical Co. Iodotubercidin (ITC), an inhibitor of AMPK, was obtained from RBI Inc. or was synthesized by Drs Alan Paterson and Wendy Gati, Department of Pharmacology, University of Alberta. Acetyl CoA was from Boehringer Mannheim. Anti AMPK α2 catalytic subunit antibody was a gift from Dr. D.G. Hardie (Scotland, U.K). All other chemicals and materials were obtained as described in the "Methods".

Heart Perfusions:

Seven day old New Zealand White rabbit hearts were perfused in the working mode at a preset pressure of 7.5 mm Hg preload and 30 mm Hg afterload, as described in the "Methods". Hearts were perfused with Krebs-Henseleit solution containing 0.4 mM [1-^{14}C]palmitate, 11 mM glucose, 3 mM calcium and 0.5 mM EDTA. Three groups of 7-day old rabbits were used for this study (Figure 6-1), as described below.

Protocol 1:

Isolated working hearts were perfused for a 30 min aerobic perfusion. Adenine nucleotide and CoA esters levels were determined at the end of the aerobic perfusion.

Protocol 2:

Isolated working hearts were perfused for a 30 min aerobic perfusion followed by 40 min of global no flow ischemia. This protocol allowed us to determine adenine nucleotide and CoA ester levels at the end of ischemia and to determine if ischemia altered ACC and AMPK protein content and/or activities.

Protocol 3:

Isolated working hearts were subjected to a 30 min aerobic perfusion followed by 40 min global no flow ischemia. This was then followed by 40 min of reperfusion in the presence or absence of AICAR (200 μM), iodotubercidin (50 μM), or 2'deoxyadenosine (300 μM). These compounds were added to the perfusate at the beginning of the perfusion protocol. This protocol allowed us to evaluate the effect of AMPK inhibition or activation on fatty acid oxidation and functional recovery during reperfusion following ischemia in the newborn hearts.

At the end of all perfusions, ventricles were quickly frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen and stored at – 80 ^{0}C until analysis.

Tissue work up:

Powdered ventricular tissues were prepared as described in the "Methods". This tissue was used to prepare PEG 8000 extracts for AMPK and ACC proteins, as described in the "Methods".

Measurement of AMP-activated protein kinase and acetyl CoA carboxylase activity:

AMPK activity determination in 6% PEG 8000 extracts was carried out as described in the "Methods".

Acetyl CoA carboxylase assay:

ACC activity determination in 6% PEG 8000 extracts was carried out by the $^{14}CO_2$ fixation assay as described in the "Methods".

Western blot analysis of ACC and AMPK:

To determine the effect of ischemia on ACC and AMPK proteins at the end of ischemia, 6% PEG 8000 extracts obtained from hearts in protocols 1 and 2 were subjected to western blot analysis on 5% and 9% SDS-PAGE, respectively, as described in the "Methods".

Determination of CoA esters and nucleotide levels:

Approximately 400 mg portion of the powdered ventricular tissue obtained from perfused hearts from protocols 1, 2 and 3 were used for 6% perchloric acid (PCA) extraction using pestle and mortar followed by HPLC analysis, as described in the "Methods".

Statistical analysis:

Results are presented as the mean ± standard error of the mean. Comparisons between individual group means were performed using the unpaired Student's t-test. In experiments involving three or more groups, analysis of variance followed by Neuman-Keuls *post-hoc* test was used to determine statistical significance between groups. Where appropriate multivariate analysis of variance was employed to determine significant difference between groups. Statistical level of significance was set at $p < 0.05$.

Results:

AMPK and ACC in aerobic and ischemic hearts:

Shown in Figure 6-2 is AMPK activity in newborn hearts at the end of the 30 min aerobic perfusion (Protocol 1) and at the end of the 30 min aerobic perfusion followed by 40 min no flow ischemia (Protocol 2). AMPK activity in the absence of AMP was significantly increased at the end of ischemia compared to aerobic values (Figure 6-2). This increase in AMPK activity was also observed when AMPK activity was measured in the presence of 200 μM AMP. Measurement of ACC activity at the end of protocols 1 and 2 showed that ACC activity was significantly decreased at the end of ischemia compared to values obtained at the end of the aerobic perfusion period (Figure 6-3).

Effect of ischemia on AMPK and ACC protein content in 7-day old hearts:

Shown in Figure 6-4A is a western blot analysis of the 63 kDa AMPK catalytic subunit from tissue obtained at the end of the 30 min aerobic perfusion and at the end of the 30 min aerobic perfusion followed by 40 min ischemia. AMPK abundance was not appreciably different in aerobic and ischemic hearts. Moreover, ACC protein content of newborn hearts in both groups was not significantly altered (Figure 6-4B).

Mechanical function in 7-day old rabbit hearts subjected to ischemia-reperfusion protocol in the presence or absence of AMPK modulators:

Shown in Table 6-1 is the effect of AICAR (200 μM), iodotubercidin (50 μM) and 2'deoxyadenosine (300 μM) on indices of mechanical function in aerobic and reperfused-ischemic hearts. Heart rate and peak systolic pressure were similar to values previously obtained under normal aerobic perfusion conditions (Chapter 5). In the reperfused ischemic period, heart rate recovered to greater than 70% of aerobic values in all groups. The peak systolic pressure was also depressed in control and AICAR-treated hearts compared to their aerobic values (Table 6-1). However, peak systolic pressure recovered to greater than 90% of pre-ischemic values in both iodotubercidin and 2'deoxyadenosine-treated hearts (Table 6-1). It is of interest that HRxPSP in AICAR-treated hearts was not significantly different from control during reperfusion following ischemia. It should be remembered, however, that HRxPSP is a poor index of cardiac mechanical function.

AMPK and ACC activity at the end of reperfusion in 7-day old rabbit hearts perfused in the presence or absence of AMPK modulators:

Shown in Figure 6-5 is AMPK activity at the end of reperfusion in control, AICAR-treated, iodotubercidin-treated and 2'deoxyadenosine-treated hearts. In the presence of 200 μM AMP, AMPK activity in AICAR-treated heart was not significantly increased compared to controls. However, both iodotubercidin and 2-deoxyadenosine-treated hearts had a significantly decreased AMPK activity compared to control hearts. Shown in Figure 6-6 is ACC activity measured at the end of the reperfusion period. AICAR significantly decreased ACC activity compared to controls. However, ACC activity in both iodotubercidin and 2'deoxyadenosine-treated hearts was significantly increased compared to controls.

Palmitate oxidation during reperfusion of 7-day old rabbit hearts subjected to ischemia-reperfusion protocol in the presence or absence of AMPK modulators:

Shown in Figure 6-7 are rates of palmitate oxidation in control, AICAR-treated, iodotubercidin-treated and 2'deoxyadenosine-treated hearts during reperfusion following ischemia. Rates of palmitate oxidation significantly increased in AICAR-treated hearts compared to controls. However, rates of palmitate oxidation in both iodotubercidin-treated and 2'deoxyadenosine-treated hearts decreased significantly compared to controls (Figure 6-7).

Cardiac work during aerobic and reperfusion of 7-day old hearts subjected to the ischemia-reperfusion protocol in the presence or absence of AMPK modulators:

Shown in Figure 6-8 is cardiac work during aerobic perfusion and during reperfusion of 7-day old hearts following ischemia. In the aerobic period, cardiac work was stable for 30 min in all groups. Moreover, cardiac work in control, AICAR-treated and iodotubercidin-treated hearts was not significantly different.

However, in 2'deoxyadenosine treated hearts an increase in cardiac work was observed compared to controls. During reperfusion, cardiac work recovered to approximately 52% of aerobic values in control hearts. In hearts treated with AICAR, cardiac work recovered to only 24% of aerobic values. Interestingly, cardiac work recovered to 92% and 106% of aerobic values, respectively, in iodotubercidin-treated and 2'deoxyadenosine-treated hearts.

Malonyl CoA levels in aerobic, ischemic and reperfused-ischemic 7-day old rabbit hearts treated with AMPK modulators:

Shown in Table 6-2 are malonyl CoA levels in aerobic, ischemic and reperfused control, AICAR-treated, iodotubercidin-treated and 2'deoxyadenosine-treated hearts. Ischemia resulted in a significant decrease in malonyl CoA levels compared to aerobic hearts. AICAR-treated hearts showed a decrease in malonyl CoA levels when compared to controls at the end of the reperfusion period. However, malonyl CoA levels were significantly increased in iodotubercidin-treated and 2'deoxyadenosine-treated hearts.

Nucleotide levels in aerobic, ischemic and reperfused-ischemic 7-day old rabbit hearts treated with AMPK modulators:

Shown in Table 6-3 are the levels of AMP, ATP, and AMP/ATP in aerobic, ischemic and reperfused control, AICAR-treated, iodotubercidin-treated and 2'deoxyadenosine-treated hearts. Tissue AMP levels increased significantly by approximately 400% in ischemic hearts compared to aerobic hearts (Table 6-3).

Tissue AMP/ATP ratio was also significantly increased at the end of ischemia compared to controls. Evaluation of AMP/ATP ratios at the end of reperfusion showed that tissue AMP/ATP ratios in AICAR-treated hearts were not significantly different compared to controls (Table 6-3). Moreover, in iodotubercidin-treated and 2'deoxyadenosine-treated heart, no significant difference in AMP/ATP ratio or AMP levels was observed (Table 6-3).

Discussion:

The results presented in this study demonstrate for the first time that inhibitors of AMPK activity can improve functional recovery during reperfusion following ischemia in newborn hearts. We recently demonstrated that the depressed functional recovery in adult rat hearts following ischemia is associated with high rates of fatty acid oxidation. This high rate of fatty acid oxidation in the reperfused-ischemic adult rat hearts results from an increase in AMPK activity and a concomitant decrease in ACC activity (Kudo et al., 1995; Kudo et al., 1996). The decrease in ACC activity results from increased phosphorylation and inactivation of ACC by AMPK (Kudo et al., 1995). In the present study, ischemia resulted in a significant increase in AMPK activity which was accompanied by a decrease in ACC activity (Figures 6-2 and 6-3). Both of these effects were without significant alterations in either AMPK or ACC protein abundance (Figure 6-4).

Previous studies in newborn heart demonstrate that a significant negative correlation exists between AMPK and ACC activities. Moreover, we showed that a significant positive correlation exists between AMPK and fatty acid oxidation (Chapter 5). Increased AMPK activity at the end of ischemia (which remained high to the end of reperfusion), and the decreased ACC activity would be expected to result in an increase in fatty acid oxidation in the reperfused-ischemic hearts. If the AMPK-ACC regulatory mechanism holds true in the reperfused-ischemic newborn heart, it is reasonable to expect that iodotubercidin should inhibit AMPK, decrease fatty acid oxidation and improve functional recovery following ischemia. Conversely, stimulation of AMPK activity should worsen functional recovery following ischemia in newborn hearts. This hypothesis was tested in this study. Indeed, both iodotubercidin and 2'deoxyadenosine-treated hearts showed a significantly decreased AMPK activity, which was associated with an increased ACC activity compared to controls (Figures 6-5 and 6-6). Of interest, is that at the end of reperfusion AMPK activity increased in AICAR-treated hearts, which was accompanied by decrease in ACC activity, and an increase in rate of fatty acid oxidation. This observation in the reperfused-ischemic heart is consistent with recent findings by Merrill et al. (1997) who demonstrated that AICAR riboside increases fatty acid oxidation in rat skeletal muscle secondary to an activation of AMPK and a decrease in ACC activity. We also demonstrated previously that ACC activity is negatively correlated with fatty acid oxidation in the newborn heart (Chapter 5). ACC is an important regulator of fatty acid oxidation via its production of malonyl CoA (Saddik et al., 1993; Lopaschuk and Gamble, 1994), a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1). CPT 1 is the rate limiting-step in mitochondria uptake of activated fatty acid. Decreased ACC activity would therefore be expected to result in a decrease in malonyl CoA levels and an increase in rate of fatty acid oxidation.

As can be seen from Figure 6-7, rates of fatty acid oxidation during reperfusion were higher in AICAR-treated hearts compared to controls and was accompanied by a decrease in malonyl CoA levels (Table 6-2). Rates of fatty acid oxidation however significantly decreased in both iodotubercidin and 2'deoxyadenosine-treated hearts, compared to controls, and was accompanied by an increase in malonyl CoA levels (see Figure 6-7 and Table 6-2). Interestingly, cardiac work during reperfusion of newborn hearts recovered to approximately

52% of aerobic values in control hearts (Figure 6-8). This level of recovery is comparable to that previously reported by Itoi et al. (1993). Increased palmitate oxidation is associated with depressed recovery of cardiac work in AICAR-treated hearts (24% of aerobic values) (Figure 6-7 and 6-8). In contrast, in iodotubercidin-treated hearts or 2'deoxyadenosine-treated hearts (both AMPK inhibitors) the decrease in rate of fatty acid oxidation was associated with improved recovery of cardiac work, which recovered to approximately 92% and 106% of their aerobic values, respectively, in iodotubercidin and 2'deoxyadenosine-treated hearts compared to controls (Figure 6-7 and 6-8). It appears that the decrease in AMPK activity and fatty acid oxidation in iodotubercidin and 2'deoxyadenosine-treated hearts resulted in an improved recovery of mechanical function, whereas increased AMPK activity and fatty acid oxidation in AICAR-treated hearts resulted in a depressed recovery of mechanical function in the reperfused-ischemic newborn hearts. Of interest, is that iodotubercidin (50 μM) also significantly improves functional recovery in adult rat heart subjected to ischemia-reperfusion (Dr. Taniguchi Masayuki, personal communication).

The mechanism by which ischemia results in activation of AMPK in the newborn heart is not fully understood. Previous studies by Hardie and colleagues in rat liver showed that increased cellular AMP levels can result in an increased AMPK activity (Corton et al., 1994; Corton et al., 1995; Davies et al., 1995; Hawley et al., 1995). Increases in the AMP/ATP ratio is an even more sensitive indicator of the stimulation of AMPK activity (Wilson et al., 1996; Davies et al., 1995). AMP can trigger a direct allosteric activation of AMPK in rat liver. Moreover, AMP can also activate an upstream AMPK kinase, resulting in phosphorylation and activation of AMPK. In addition, increased AMP levels can promote phosphorylation of AMPK by the upstream AMPK kinase and inactivate protein phosphatase 2A. This prevents the dephosphorylation and inactivation of AMPK (Weekes et al., 1994; Davies et al., 1995). The ultimate result is an AMPK with a preserved phosphorylation status and high catalytic activity. In this study, ischemia resulted in both an increase in AMP levels and an increase in the AMP/ATP ratio compared to aerobic hearts (Table 6-3), which was accompanied by an activation of AMPK activity. The AMP/ATP ratio also increased in AICAR treated hearts at the end of reperfusion (Table 6-3). However, neither iodotubercidin nor 2'deoxyadenosine-treated hearts showed significant alteration in cellular AMP or the AMP/ATP ratio (Table 6-3). However, AMPK activity in both groups significantly decreased compared to controls (Figure 6-5). It therefore appears that AMPK inhibitors act by a direct interaction with the AMPK protein, rather than by direct changes in cellular AMP or AMP/ATP ratios.

The mechanism by which increased fatty acid oxidation following ischemia results in a depressed functional recovery is not fully understood. Earlier studies by Lopaschuk et al. (1992) suggested that high rates of fatty acid oxidation inhibit rates of glucose oxidation. This decrease in glucose oxidation can lead to an imbalance between rates of glycolysis and glucose oxidation, resulting in proton production and acidosis. The excessive proton production results from an increased hydrolysis of glycolytically derived ATP. Excessive cellular acidosis can result in altered Na/H$^+$ exchanger activity, accumulation of sodium in the cytosol, ultimately leading to calcium overload. This condition may result in both damage to cardiac myocytes and to a depression in contractile function (Neely and Grotyohann, 1984; Tani and Neely, 1989; Tani, 1992; Dennis et al., 1990).

This explanation appears attractive since tricarboxylic acid cycle activity and ATP production resumes during reperfusion following ischemia (Liu et al., 1996) and it is unlikely that the poor recovery of mechanical function following ischemia results from non availability of ATP for the contractile machinery of cardiac myocytes. It is conceivable that AMPK inhibitors by decreasing rate of fatty acid oxidation may indirectly increase glucose oxidation and improve the coupling of glycolysis to glucose oxidation. The role of AMPK inhibitors in decreasing proton production during and following ischemic episode remains to be investigated.

In summary, we demonstrate that AMPK activity increases during ischemia and remains elevated during reperfusion in control hearts. This increased AMPK activity results in a decrease in ACC activity and malonyl CoA levels during reperfusion, which was accompanied by both an increased rate of myocardial fatty acid oxidation and poor functional recovery. Furthermore, we demonstrate that AMPK inhibitors improve the recovery of cardiac function following ischemia, compared to control hearts. This improved functional recovery is due to decrease in rate of fatty acid oxidation, secondary to an increase in ACC activity and malonyl CoA levels in the reperfused-ischemic hearts. We therefore propose that AMPK is an important regulator of fatty acid oxidation in the reperfused-ischemic hearts. A schematic representation of how AMPK modulators alter fatty acid oxidation to significantly affect functional recovery during reperfusion is shown in Figure 6-9. It is possible that results of this pre-clinical study with AMPK inhibitors may be reproducible in the clinical setting of ischemia-reperfusion. In that instance, AMPK inhibitors may improve functional recovery following cardiac surgery in infants and newborn undergoing open heart surgery to correct congenital heart defects.

Table 6-1:

Effect of AICAR (200 μM) iodotubercidin (50 μM) and 2'deoxyadenosine (300 μM) on mechanical function in aerobic and reperfused-ischemic 7-day old newborn hearts.

Perfusion Condition	HR (beats /min)	PSP (mmHg)	DP (mmHg)	HR x PSP (x 10^{-3})
Aerobic				
Control	220 ± 10	40 ± 2	35 ± 2	8.8 ± 1.6
AICAR	210 ± 5	45 ± 3	36 ± 3	9.4 ± 2.0
ITC	190 ± 10	45 ± 1	34 ± 3	8.5 ± 1.5
DeoxyADO	200 ± 5	53 ± 2*	37 ± 2	10.5 ± 1.6
Reperfused-ischemic				
Control	195 ± 2	25 ± 4	20 ± 3	4.9 ± 0.8
AICAR	145 ± 3	18 ± 2	15 ± 1	2.6 ± 0.5
ITC	200 ± 2	41 ± 2*	35 ± 2*	8.1 ± 0.9*
DeoxyADO	220 ± 3	53 ± 3*	38 ± 2*	11.6 ± 1.8*

Values are the mean ± SEM of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

* significantly different from control hearts in comparable group.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine.

Table 6.2:

Effect of ischemia, AICAR (200 μM) iodotubercidin (50 μM) and 2'deoxyadenosine (300 μM) on CoA ester levels at the end of perfusion in aerobic and in reperfused-ischemic 7-day old newborn hearts.

Perfusion Condition	**Malonyl CoA** (nmol / g dry wt)
Aerobic	5.2 ± 0.8
Ischemia	2.3 ± 0.4†
Reperfused-ischemic	
Control	3.5 ± 0.6
AICAR	1.6 ± 0.4
ITC	13.3 ± 1.9*
DeoxyADO	11.7 ± 1.2*

Values are the mean ± SEM of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

† significantly different from aerobic hearts.

* significantly different from control hearts.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine

Table 6.3:

Effect of ischemia, AICAR (200 μM), iodotubercidin (50 μM) and 2'deoxyadenosine (300 μM) on nucleotide levels at the end of perfusion in aerobic and in reperfused-ischemic 7-day old newborn hearts

Perfusion Condition	**ATP** (μmol / g dry wt)	**AMP** (μmol / g dry wt)	**AMP/ATP Ratio**
Aerobic	16.5 ± 0.8	3.8 ± 0.6	0.23 ± 0.02
Ischemia	10.6 ± 0.6†	16.6 ± 1.4†	1.57 ± 0.09†
Reperfused-ischemic			
Control	17.6 ± 1.6	4.6 ± 0.6	0.32 ± 0.08
AICAR	18.6 ± 1.4	10.6 ± 0.7	0.57 ± 0.11
ITC	20.6 ± 1.7	4.4 ± 0.9	0.31 ± 0.06
DeoxyADO	16.6 ± 0.5	4.5 ± 0.7	0.28 ± 0.12

Values are the mean ± SEM of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

† significantly different from aerobic hearts.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine.

Figure 6-1:

Schematic diagram showing three different perfusion protocols used in the study as described in Methods section of this chapter.

Protocols

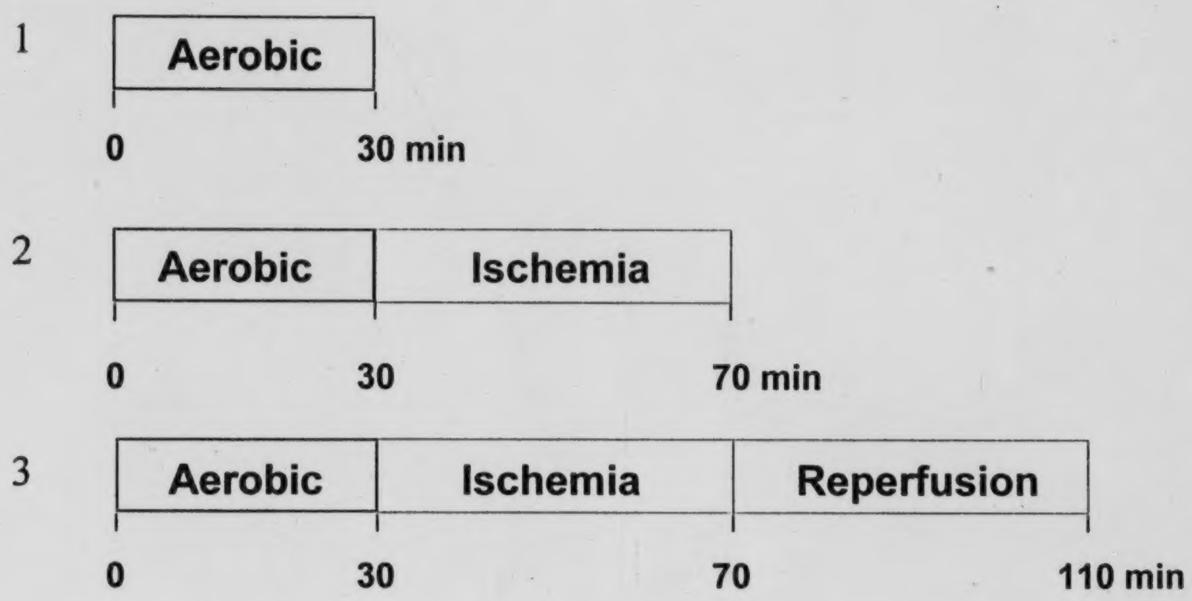

1
Aerobic
0
30 min
2
Aerobic
Ischemia
0
30
70 min
3
Aerobic
Ischemia
Reperfusion
0
30
70
110 min

Figure 6-2:

AMPK activity in 6% PEG 8000 extracts from 7-day old newborn rabbit hearts obtained at the end of aerobic and at the end of ischemia.

AMPK activity in 6% PEG 8000 extracts was determined in the presence or absence of 200 μM AMP as described in Methods.

Values are the mean ± S.E.M. of results from 8 aerobic and 7 ischemia hearts.

* significantly different from 0 AMP

† significantly different from aerobic hearts.

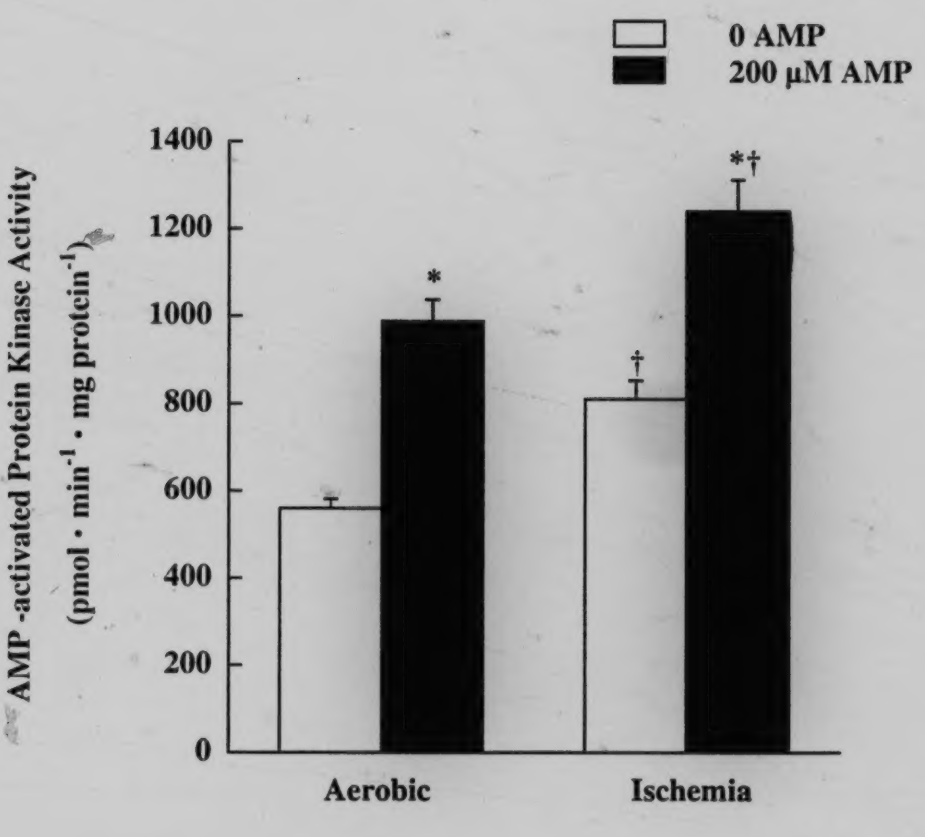

0 AMP
200 µM AMP
AMP -activated Protein Kinase Activity
(pmol · min⁻¹ · mg protein⁻¹)
1400
1200
1000
800
600
400
200
0
*
†
*†
Aerobic
Ischemia

Figure 6-3:

ACC activity in 6% PEG 8000 extracts from 7-day old newborn rabbit hearts obtained at the end of aerobic and at the end of ischemia.

ACC activity in 6% PEG 8000 extracts was determined by the $^{14}CO_2$ fixation assay as described in Methods.

Values are the mean ± S.E.M. of results from 8 aerobic and 7 ischemia hearts.

* significantly different from aerobic hearts.

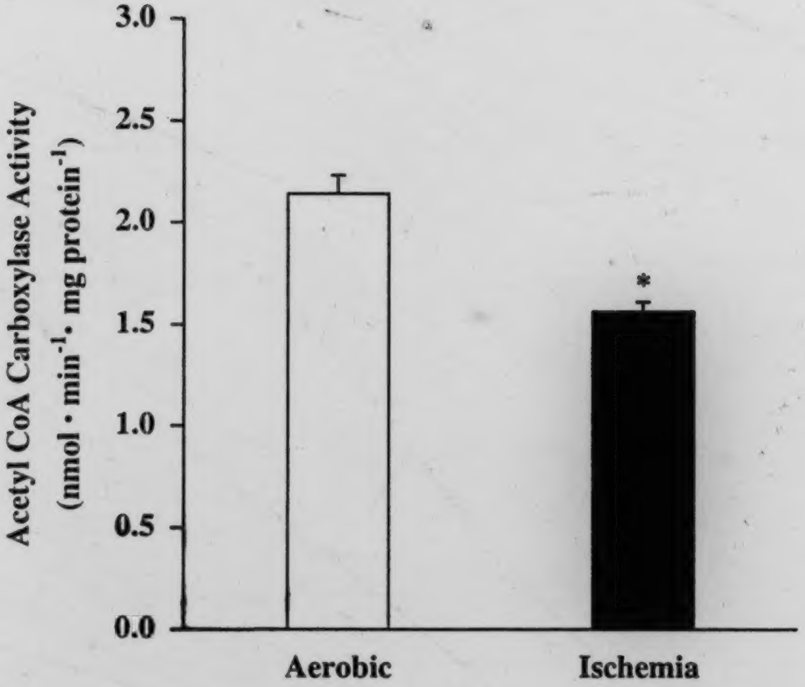

Acetyl CoA Carboxylase Activity
(nmol · min-1 · mg protein-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Aerobic
Ischemia

Figure 6-4:

Representative immunoblot analysis showing (A) AMPK and (B) ACC protein levels in 6% PEG 8000 extracts from 7-day old newborn rabbit hearts obtained at the end of aerobic and at the end of ischemia.

Immunoblot analysis of AMPK and ACC proteins were carried out on a 9% and 5% SDS-PAGE, respectively, as in Methods.
Ischemia did not result in changes in AMPK or ACC protein abundance.

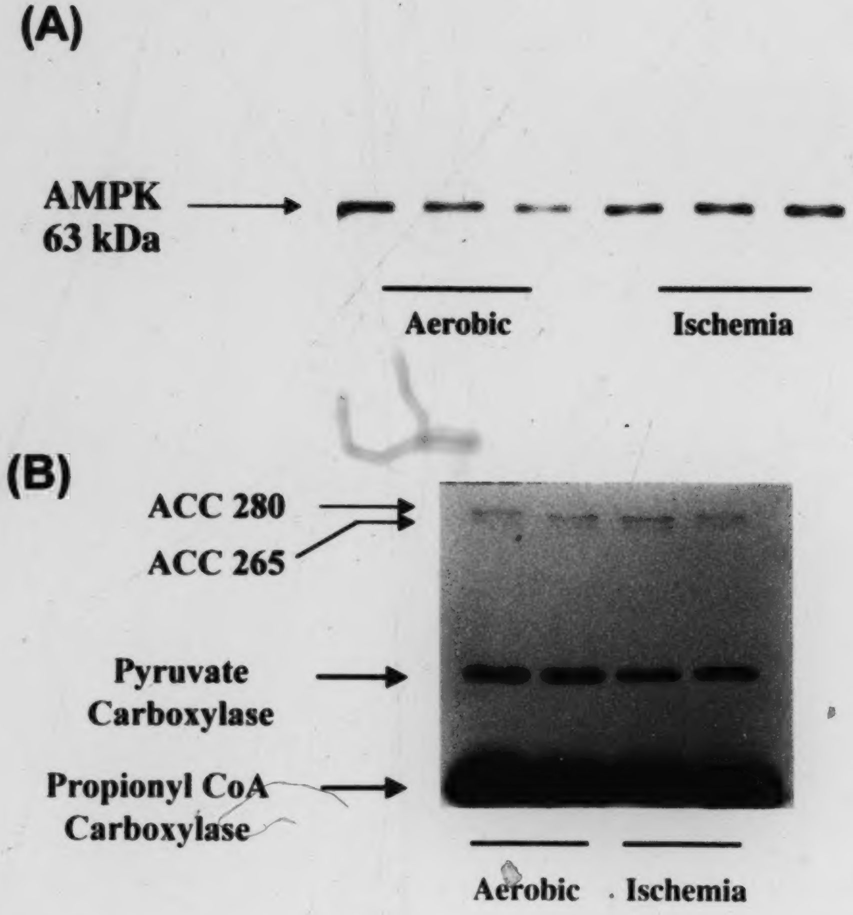

(A)
AMPK
63 kDa
Aerobic
Ischemia
(B)
ACC 280
ACC 265
Pyruvate Carboxylase
Propionyl CoA Carboxylase
Aerobic
Ischemia

Figure 6-5:

AMPK activity in 6% PEG 8000 extracts obtained from control, AICAR, iodotubercidin, or 2'deoxyadenosine-treated 7-day old newborn hearts.

AMPK activity in 6% PEG 8000 extracts was determined in the presence of 200 μM AMP as described in Methods.

Values are the mean ± S.E.M. of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

* significantly different from control hearts.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine

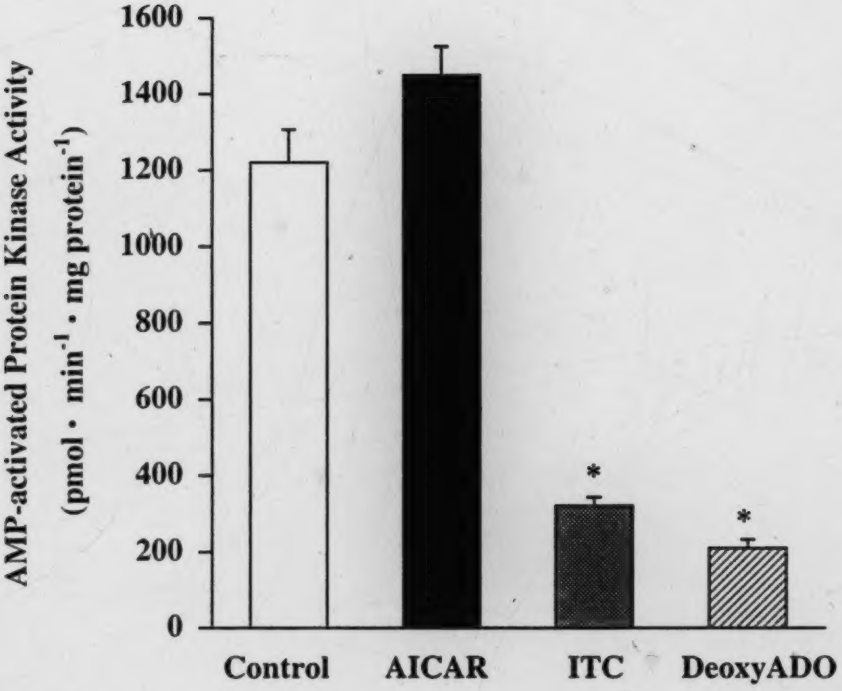

AMP-activated Protein Kinase Activity
(pmol · min^{-1} · mg protein^{-1})
1600
1400
1200
1000
800
600
400
200
0
*
*
Control
AICAR
ITC
DeoxyADO

Figure 6-6:

ACC activity in 6% PEG 8000 extracts obtained from control, AICAR (200 µM), iodotubercidin (50 µM), or 2'deoxyadenosine-treated (300 µM) 7-day old newborn hearts.

ACC activity in 6% PEG 8000 extracts was determined by the $^{14}CO_2$ fixation assay as described in Methods.

Values are the mean ± S.E.M. of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

* significantly different from control hearts.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine.

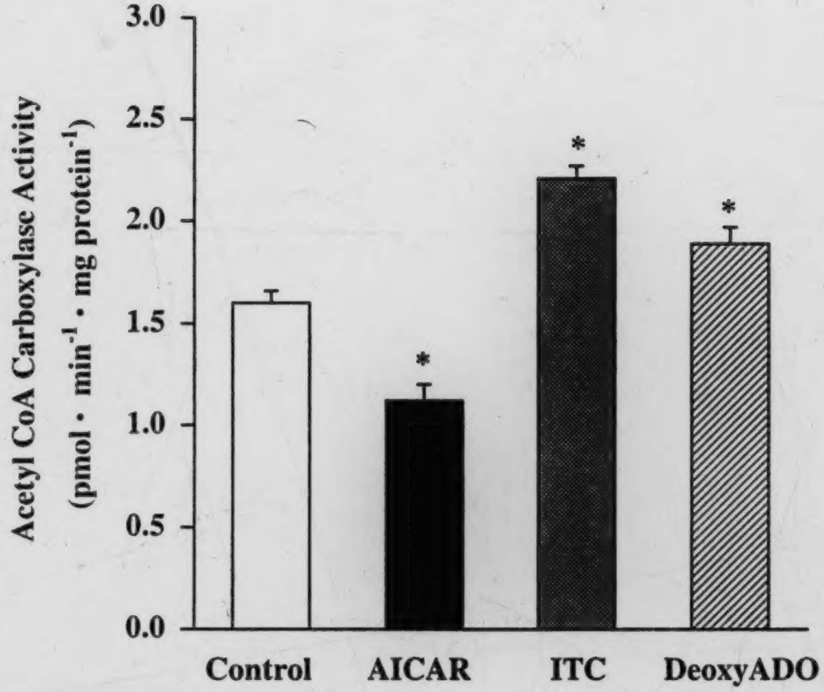

Acetyl CoA Carboxylase Activity
(pmol · min^{-1} · mg protein^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
AICAR
ITC
DeoxyADO

Figure 6-7:

Palmitate oxidation rates in control, AICAR (200 μM), iodotubercidin (50 μM), or 2'deoxyadenosine-treated (300 μM) 7-day old newborn hearts.

Palmitate oxidation rates were determined as described in Methods.

Values are the mean ± SEM of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

* significantly different from control hearts.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine.

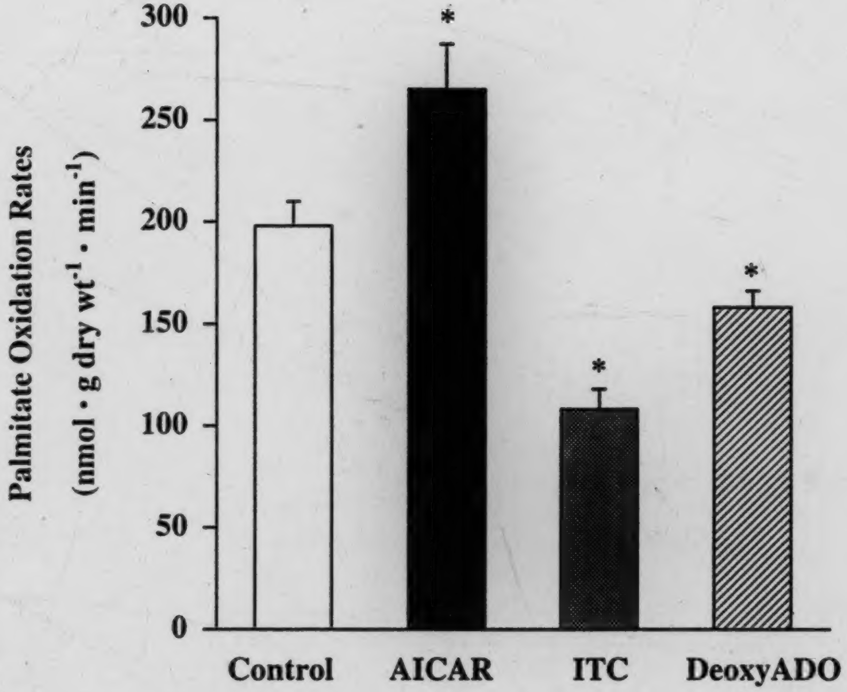

Palmitate Oxidation Rates
(nmol • g dry wt^{-1} • min^{-1})
300
250
200
150
100
50
0
Control
AICAR
ITC
DeoxyADO
*
*
*

Figure 6-8:

Cardiac work with time during aerobic, and reperfusion following ischemia in control, AICAR (200 μM), iodotubercidin (50 μM), or 2'deoxyadenosine-treated (300 μM) 7-day old newborn hearts.

Cardiac work recovered to greater than 90% of pre-ischemic values in iodotubercidin and 2'deoxyadenosine-treated hearts. AICAR-treated hearts however recovered to approximately 24% of aerobic values.

Values are the mean ± SEM of results from 6 control, 5 AICAR, 6 iodotubercidin and 6, 2'deoxyadenosine-treated hearts.

* significantly different from control hearts.

ITC = iodotubercidin; DeoxyADO = 2'deoxyadenosine.

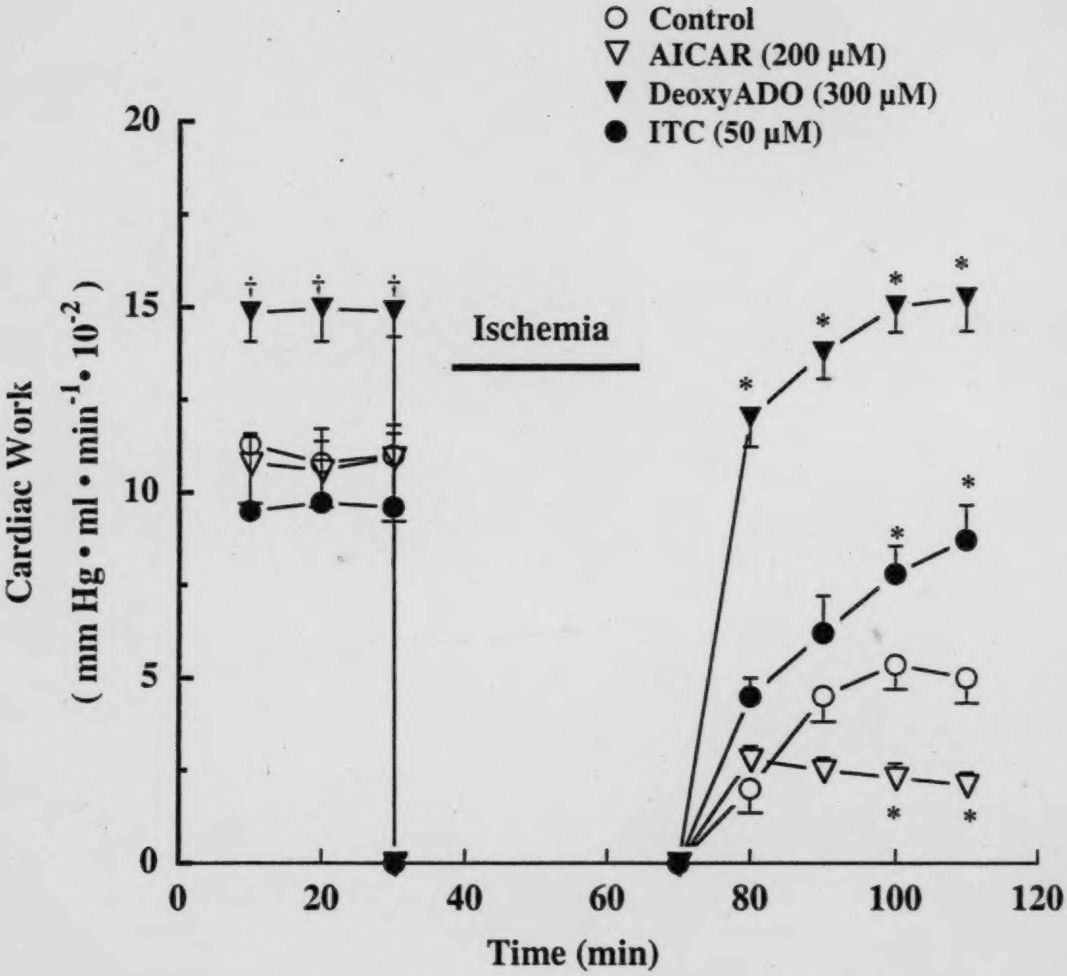
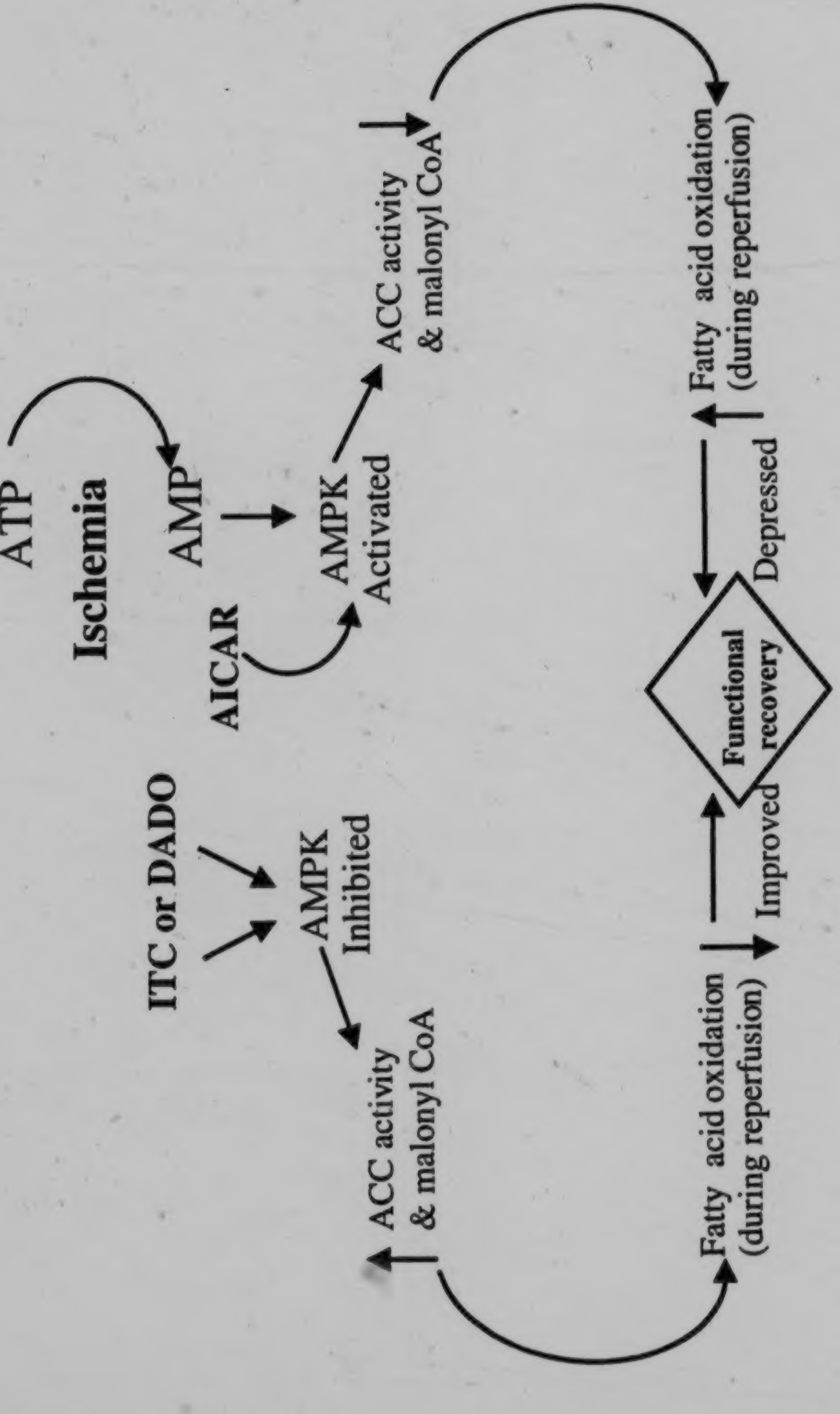

○ Control
▽ AICAR (200 µM)
▼ DeoxyADO (300 µM)
● ITC (50 µM)
Ischemia
Cardiac Work
(mm Hg • ml • min^{-1} • 10^{-2})
Time (min)
0
5
10
15
20
0
20
40
60
80
100
120
†
*

Figure 6-9:

Proposed scheme showing the mechanism by which AMPK activation or inhibition, respectively, results in a depressed or improved functional recovery in reperfused-ischemic newborn heart.

The increase in cytosolic AMP levels during ischemia stimulates AMPK catalytic activity, resulting in an increased phosphorylation of ACC (ACC activity will decrease under this condition). This in turn results in lower malonyl CoA levels and a stimulation of rate of fatty acid oxidation secondary to an increase in carnitine palmitoyltransferase 1 (CPT 1) activity. Increased rate of fatty acid oxidation results in depressed functional recovery following ischemia. Conversly, inhibition of AMPK activity by iodotubercidin or 2'deoxyadenosine prevents (or decreases) the progression of the series of events described above. The resultant decrease in rate of fatty acid oxidation results in improved functional recovery following ischemia as described in text.


ATP
Ischemia
AMP
AICAR
AMPK
Activated
ACC activity
& malonyl CoA
Fatty acid oxidation
(during reperfusion)
Depressed
Functional
recovery
Improved
ITC or DADO
AMPK
Inhibited
ACC activity
& malonyl CoA
Fatty acid oxidation
(during reperfusion)

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Kudo, N., Gillespie, J.G., Kung, L., Witters, L.A., Schulz, R., Clanachan, A.S., and Lopaschuk, G.D. (1996) Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl CoA carboxylase during reperfusion following ischemia. *Biochim. Biophy. Acta* **1301**, 67-75.

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Lopaschuk, G.D., Wambolt, RB., and Barr, R.L. (1992) An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. *J. Pharmacol. Exp. Ther.* **264,** 135-144.

Lopaschuk, G.D., Witters, L.A., Itoi, T., Barr, R., and Barr, A. (1994b) Acetyl CoA carboxylase involvement in the rapid maturation of fatty acid oxidation in the newborn rabbit heart. *J. Biol. Chem.***269**, 25871-25878.

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Merrill, G.F., Kurth, E.J., Hardie, D.G., and Winder W.W. (1997) AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am. J. Physiol.* **273,** E1107-E1112.

Moore, F., Weekes, J., and Hardie, D.G. (1991) Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. *Eur. J. Biochem.* **199**, 691-697.

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Weekes, J., Hawley, S.A., Corton, J., Shugar, D., and Hardie, D.G. (1994) Activation of rat liver AMP-activated protein kinase by kinase kinase in a purified, reconstitued system. *Eur. J. Biochem.* **219,** 751-757.

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CHAPTER 7

Glucagon and isoproterenol stimulate fatty acid oxidation in newborn rabbit heart by 5'AMP-activated protein kinase independent mechanism.

Introduction

We demonstrated recently that AMP-activated protein kinase (AMPK) is present in newborn heart with measurable activity (Chapters 3 & 4). This newborn heart AMPK consists of a 63 kDa α catalytic subunit and both 48 kDa β and 38 kDa γ regulatory subunits (Chapter 3). We have also shown that increased AMPK activity results in inactivation of acetyl CoA carboxylase (ACC), an enzyme which regulates fatty acid oxidation in the heart via its production of malonyl CoA (Chapter 5). However, in rat liver, ACC can also be phosphorylated and inactivated by cyclic AMP dependent protein kinase (PKA), a kinase which is activated by glucagon (Holland et al., 1984). Since circulating blood glucagon levels rise in the immediate post-natal life (Girard et al., 1992), and fatty acid oxidation increases within the same period in 7-day compared to 1-day old hearts (Lopaschuk and Spafford, 1990), the possibility exists that glucagon may contribute to increased fatty acid oxidation in 7-day old hearts. To date, the role of increased blood glucagon levels in the increase in myocardial fatty acid oxidation following birth remains to be determined.

Earlier studies by Holland et al. (1984), Swenson and Porter (1985) and Mabrouk et al. (1990) suggested that glucagon may decrease ACC activity via activation of protein kinase A in rat hepatocytes. Sim and Hardie (1988), however suggested that glucagon decreases ACC activity via stimulation of AMPK. Since glucagon decreases ACC activity in these studies, it is possible that glucagon stimulates fatty acid oxidation in 7-day old hearts secondary to a decrease in ACC activity and malonyl CoA levels. Whether this decrease in ACC activity results from activation of AMPK or a stimulation of PKA in the 7-day old heart remains to be determined. We hypothesized that glucagon stimulates fatty acid oxidation and inactivates ACC secondary to activation of AMPK in 7-day old hearts.

The purpose of this study is to determine if glucagon stimulates AMPK, and therefore decreases ACC activity and malonyl CoA levels in 7-day old hearts. In addition, we determined whether this is accompanied by an increase in myocardial fatty acid oxidation. To achieve this purpose, 7-day old rabbit hearts were perfused with Krebs-Henseleit solution containing ^{14}C-palmitate in the presence or absence of glucagon (1 ng/ml). Fatty acid oxidation and AMPK activity were determined. Moreover, we determined if the effect of glucagon in the newborn heart was associated with an increase in cyclic AMP levels. Furthermore, the effect of another inotrope, isoproterenol (which increases cyclic AMP levels) on both fatty acid oxidation and AMPK activity was determined. Our data demonstrate that both glucagon and isoproterenol increase myocardial fatty acid oxidation in the 7-day old heart by an AMPK independent mechanism.

Experimental Procedures:

Materials

All materials used in this study were obtained as described in Methods (Chapter 2). Anti AMPK α1 and α2 catalytic subunit antibodies were a gift from Dr. D. G. Hardie, Dundee, U.K.

Heart perfusions

Hearts from seven day old New Zealand White rabbits were used in this study. Hearts were perfused with Krebs-Henseleit solution containing 11 mM glucose, 0.4 mM [1-^{14}C]palmitate in the presence or absence of glucagon (1 ng/ml) and in the presence or absence of isoproterenol (3×10^{-8} M) for a 40 min aerobic period at a preload pressure of 7.5 mm Hg and an afterload pressure of 30 mm Hg as described in Methods.

Tissue work up

Following heart perfusions the ventricles were quickly frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen and stored at – 80 ^{0}C until ready for analysis.

Heart extractions and measurement of 5'AMP activated protein kinase and acetyl CoA carboxylase activities

Approximately 200 mg of frozen ventricular tissue was used to prepare PEG 8000 extracts. AMPK and ACC activities were then measured as described in Methods.

Western blot analysis of AMPK and ACC

To determine if glucagon had any effect on AMPK or ACC protein expression, samples of 6% PEG 8000 extracts obtained from control and glucagon-treated hearts were subjected to 9% and 5% SDS-PAGE for AMPK (α1 or α2 catalytic subunits) and ACC proteins, respectively. Chemiluminescence detection was then performed to visualize AMPK and ACC proteins as described in Methods.

Determination of CoA esters and nucleotide levels in glucagon and isoproterenol treated hearts

Adenine nucleotide levels and coenzyme A esters content of perfused heart ventricular tissues were determined as previously described in Methods.

Determination of cyclic AMP levels in glucagon and isoproterenol treated hearts

Cyclic AMP levels in perfused heart tissues were determined by using a cAMP assay kit (Cayman Chemical Co.) as previously outlined in Methods.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Comparisons between two group means were performed using an unpaired Student's t-test. Where more than two group means were compared, analysis of variance followed by the Neuman-Keul's post-hoc test was used to determine if a significant difference existed between groups. Statistical significance was set at $p < 0.05$.

Results:

Mechanical and hemodynamic function in glucagon and isoproterenol perfused newborn rabbit hearts

Table 7-1 summarizes the results of the effects of glucagon and isoproterenol on indices of both mechanical and hemodynamic function in 7-day old rabbit hearts. As expected, glucagon increased heart rate, peak systolic pressure, cardiac output, developed pressure and cardiac work. Isoproterenol also had similar effects as glucagon on mechanical and hemodynamic functions.

Palmitate oxidation in newborn hearts perfused with glucagon or isoproterenol

Shown in Figure 7-1 is the effect of glucagon or isoproterenol on the rate of palmitate oxidation in isolated working newborn rabbit hearts. Glucagon significantly increased the rate of palmitate oxidation compared to controls. The rate of palmitate oxidation was also increased in isoproterenol-treated 7-day old rabbit hearts compared to controls.

Effect of glucagon and isoproterenol on AMPK activity in the newborn rabbit heart

Shown in Figure 7-2 is AMPK activity in 7-day old hearts perfused in the presence and absence of glucagon (1 ng/ml) or isoproterenol ($3x10^{-8}$ M).

Glucagon did not have any significant effect on AMPK activity in the presence or absence of 200 μM AMP. Also, when hearts were perfused with isoproterenol, AMPK activity was not significantly altered. This result in the newborn heart differed from the results of studies by Sim and Hardie (1988) in rat hepatocytes, where glucagon had stimulated AMPK activity.

Effect of glucagon or isoproterenol on ACC activity and malonyl CoA levels in newborn rabbit hearts

We determined if glucagon or isoproterenol had any effect on ACC activity in newborn hearts. As shown in Figure 7-3, both glucagon and isoproterenol decreased ACC activity in isolated newborn rabbit hearts. Also shown in Figure 7-4 is the level of malonyl CoA in hearts perfused with glucagon or isoproterenol. Both glucagon and isoproterenol significantly decreased malonyl CoA levels compared to controls. This decrease in malonyl CoA levels suggests a relieving of the inhibitory effects of malonyl CoA on CPT 1 activity.

Effect of glucagon on the protein levels of both α1 and α2 catalytic subunits of the AMPK and ACC in newborn hearts

Shown in Figure 7-5A is the effect of glucagon on both isozymes of the AMPK catalytic subunit. Neither α1 nor α2 catalytic subunits of AMPK were significantly altered in glucagon-treated hearts compared to controls. Moreover, there was no significant alteration in ACC protein content in glucagon-treated hearts compared to controls (Figure 7-5B).

Effect of glucagon or isoproterenol on nucleotide levels in newborn hearts

Shown in Table 7-2 is the effect of glucagon or isoproterenol on AMP and ATP levels, as well as the AMP/ATP ratio in 7-day old hearts. Neither glucagon nor isoproterenol significantly altered AMP or ATP levels compared to controls. Moreover, the AMP/ATP ratio was not significantly different compared to controls. This finding further supports the notion that glucagon and isoproterenol may not be altering ACC activity via activation of AMPK, but possibly through activation of other kinases such as the cyclic AMP dependent protein kinase.

Effect of glucagon or isoproterenol on cAMP levels in newborn hearts

To determine if the decrease in ACC activity by glucagon was secondary to PKA activation, cyclic AMP levels were measured in 7-day old hearts treated with glucagon or isoproterenol. As shown in Figure 7-6, both glucagon and isoproterenol significantly increased cyclic AMP levels compared to controls. This increase in cAMP levels is consistent with an activation of PKA in newborn heart by glucagon or isoproterenol.

Discussion

The first important finding of this study is that both glucagon and isoproterenol stimulate palmitate oxidation in 7-day old hearts compared to controls (Figure 7-1). This increase in palmitate oxidation was secondary to inactivation of ACC. However, contrary to previous reports by Sim and Hardie (1988) in isolated rat hepatocytes, which demonstrated a decreased ACC activity secondary to an activation of AMPK by glucagon, in our hands, glucagon-induced a decrease in ACC activity which was not associated with an increase in AMPK activity (Figure 7-2). In agreement with Sim and Hardie (1988), however, glucagon significantly decreased ACC activity in 7-day old hearts (Figure 7-3).

The importance of ACC in the regulation of fatty acid metabolism in the adult rat heart has recently been established (Saddik et al., 1993; Lopaschuk and Gamble, 1994; Kudo et al., 1995; Kudo et al., 1996). Decreased ACC activity results in decreased malonyl CoA levels. Malonyl CoA itself is the end product of a two stage carboxylation of acetyl CoA by ACC and is a potent inhibitor of carnitine palmitoyltransferase 1 activity, the rate limiting enzyme in the intra-mitochondrial uptake of activated fatty acids (McGarry et al., 1978). As shown in Figure 7-4, malonyl CoA levels decrease significantly in both glucagon and isoproterenol-treated hearts. This decrease in malonyl CoA levels may be expected to relieve the inhibitory effects of malonyl CoA on CPT 1 and to facilitate the uptake and oxidation of activated fatty acids.

Although it is clear that glucagon decreased ACC activity independent of AMPK activation, it is not known how glucagon inhibited ACC activity in 7-day old hearts. It is of interest, however, that glucagon did not alter either ACC or

AMPK protein content (Figure 7-5A & B) of perfused 7-day old hearts. One possibility, therefore, is that glucagon activated cyclic AMP dependent protein kinase, which phosphorylates and inactivates ACC as previously demonstrated in the rat liver (Holland et al., 1984). As shown in Figure 7-6, glucagon increased cyclic AMP levels. This result is, therefore, in support of the notion that glucagon increases palmitate oxidation by decreasing ACC activity via activation of PKA.

To confirm this observation, another series of hearts was perfused with isoproterenol, a β-adrenergic receptor agonist known to increase cyclic AMP levels. Like glucagon, isoproterenol decreased ACC activity, which was accompanied by an increase in the rate of palmitate oxidation compared to controls (Figures 7-1 and 7-3). These results are consistent with earlier reports by Haystead et al. (1990) which demonstrated that isoproterenol decreased ACC activity in rat adipocytes secondary to an increase in cyclic AMP levels. It is also in agreement with recent studies in our laboratory which show that PKA activation (as may be the case with glucagon or isoproterenol) can directly result in phosphorylation and inactivation of rat heart ACC (Dyck et al., 1998). It is of interest, however, that isoproterenol treatment of newborn hearts also did not result in any significant activation of AMPK activity (Figure 7-2). These results are, therefore, consistent with the recent report by Hall et al. (1996) which showed that the dobutamine-induced increase in fatty acid uptake was not accompanied by an increase in AMPK activity. It appears that both glucagon and isoproterenol decreased ACC activity and malonyl CoA levels via an AMPK independent mechanism in isolated perfused newborn hearts.

To further elucidate the possible reason why glucagon treatment did not result in activation of AMPK activity, we determined if glucagon altered the levels of AMP or ATP or the AMP/ATP ratio in newborn rabbit hearts. Previous studies showed that an increase in AMP levels or an increased AMP/ATP ratio (Chapter 6) can trigger the phosphorylation and activation of AMPK. Moreover, AMP can inhibit dephosphorylation of AMPK, thereby keeping AMPK in its catalytically active phosphorylated state (Henin et al., 1996; Hawley et al., 1995; Davies et al., 1995; Moore et al., 1991). As shown in Table 7-2, neither glucagon nor isoproterenol significantly altered AMP levels. In addition, the AMP/ATP ratio was not appreciably altered. This, therefore, suggests that the decrease in ACC activity seen in glucagon-treated hearts may indeed be due to mechanisms other than the activation of AMPK. Since cAMP levels increase in both glucagon and isoproterenol-treated hearts, our data strongly suggest that glucagon or isoproterenol altered the rate of fatty acid oxidation in 7-day old hearts by decreasing ACC activity secondary to an elevation of cAMP levels.

These results have implications on the maturation of fatty acid oxidation in the immediate newborn period. High insulin levels decrease AMPK activity in isolated rat hepatocytes and the newborn heart (Witters and Kemp, 1992; Chapter 4). Since circulating blood levels of insulin decrease and glucagon levels rise in the immediate newborn period (Girard et al., 1992), a decrease in circulating insulin levels may increase AMPK, decrease ACC activity and increase fatty acid oxidation. Similarly, an increase in glucagon levels may cause a rise in cyclic AMP levels with a resultant activation of PKA, which results in phosphorylation and inactivation of ACC, resulting in an increase in fatty acid oxidation. Therefore, increased glucagon levels may contribute significantly to the rapid maturation of myocardial fatty acid oxidation in the immediate newborn period, most probably by decreasing ACC activity via activation of PKA. A schematic diagram depicting this possibility is shown in Figure 7-7.

Results of our present study also demonstrate that a β-agonist like isoproterenol, possibly by a mechanism similar to that caused by glucagon, increases palmitate oxidation in 7-day old hearts. This has clinical relevance especially when consideration is given to the fact that β-agonists (and other positive inotropes) are usually employed as adjuvants in pediatric cardiac surgery to increase myocardial performance following surgery. Since high rates of fatty acid oxidation contribute to ischemic injury (Chapter 6), it is possible that the effect of isoproterenol on ACC activity and fatty acid oxidation may worsen the recovery of heart function following an ischemic episode. The role of catecholamines in the recovery of mechanical function in the reperfused-ischemic newborn rabbit heart is currently being investigated in our laboratory.

Overall, results from this study demonstrate that both glucagon and isoproterenol stimulate fatty acid oxidation in the newborn heart. Moreover, we demonstrate by several lines of evidence that this increase in fatty acid oxidation is not associated with an increase in AMPK activity. Since cAMP levels are significantly increased in these hearts it is possible that glucagon and isoproterenol increase fatty acid oxidation secondary to a decrease in ACC activity by an AMPK independent mechanism. We suggest that glucagon and isoproterenol increase fatty acid oxidation secondary to an activation of PKA.

Table 7-1:

Effect of glucagon (1 ng/ml) or isoproterenol (3 x 10^{-8} M) on mechanical and hemodynamic function in isolated working hearts from 7-day old rabbits.

Parameter Measured	Control	Glucagon	Isoproterenol
Heart Rate (beats / min)	195 ± 6	230 ± 2*	235 ± 2*
Peak Systolic Pressure (mmHg)	42 ± 1	49 ± 1*	52 ± 3*
Developed Pressure (mmHg)	36 ± 2	38 ± 3	45 ± 2*
HR x PSP (mmHg.beat / min.10^{-3})	8.2 ± 1.6	11.3 ± 1.9*	12.6 ± 1.4*
Cardiac Output (ml / min)	26 ± 2	33 ± 2*	40 ± 3*
Aortic Flow (ml / min)	10 ± 3	15 ± 2	33 ± 1*
Coronary Flow (ml / min)	16 ± 2	17 ± 5	7 ± 2*
Cardiac Work (mmHg ml /min x 10^{-2})	10.9 ± 1.5	16.8 ± 2.2*	19.2 ± 2.0*

Values are the mean ± SEM of results from 11 control hearts and 6 hearts each in glucagon and isoproterenol groups.

* significantly different from control hearts.

Table 7-2:

Effect of glucagon (1 ng/ml) or isoproterenol (3 x 10^{-8} M) on nucleotide levels in 7-day old isolated working hearts from 7-day old rabbits.

Perfusion Condition	**ATP** (μmol / g dry wt)	**AMP** (μmol / g dry wt)	**AMP/ATP Ratio**
Control	18.4 ± 0.8	4.2 ± 0.9	0.23 ± 0.08
Glucagon	24.6 ± 0.4	5.4 ± 1.3	0.21 ± 0.10
Isoproterenol	25.1 ± 1.2	6.1 ± 0.4	0.24 ± 0.06

Values are the mean ± SEM of results from 8 control hearts and 6 hearts each in glucagon and isoproterenol groups.

Figure 7-1:

Palmitate oxidation rates in 7-day old isolated rabbit hearts perfused in the presence or absence of glucagon (1 ng/ml) or isoproterenol (3×10^{-8} M).

Values are the mean ± SEM of results from 11 control hearts and 6 hearts each in glucagon and isoproterenol-treated groups.

* significantly different from control hearts.

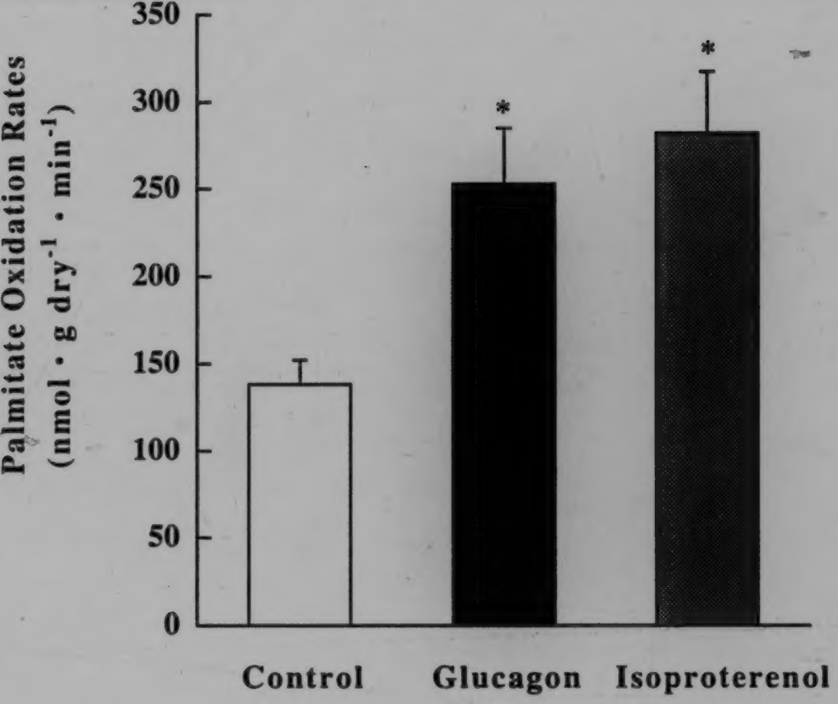

Palmitate Oxidation Rates
(nmol · g dry^{-1} · min^{-1})
350
300
250
200
150
100
50
0
*
*
Control
Glucagon
Isoproterenol

Figure 7-2:

AMPK activity in 7-day old rabbit hearts perfused in the presence or absence of glucagon or isoproterenol.

AMPK activity was measured in the presence or absence of 200 μM AMP as described in Methods.

Values shown are the mean ± SEM of results from 8 control hearts and 6 hearts each for glucagon and isoproterenol-treated groups.

* significantly different from 0 AMP hearts.

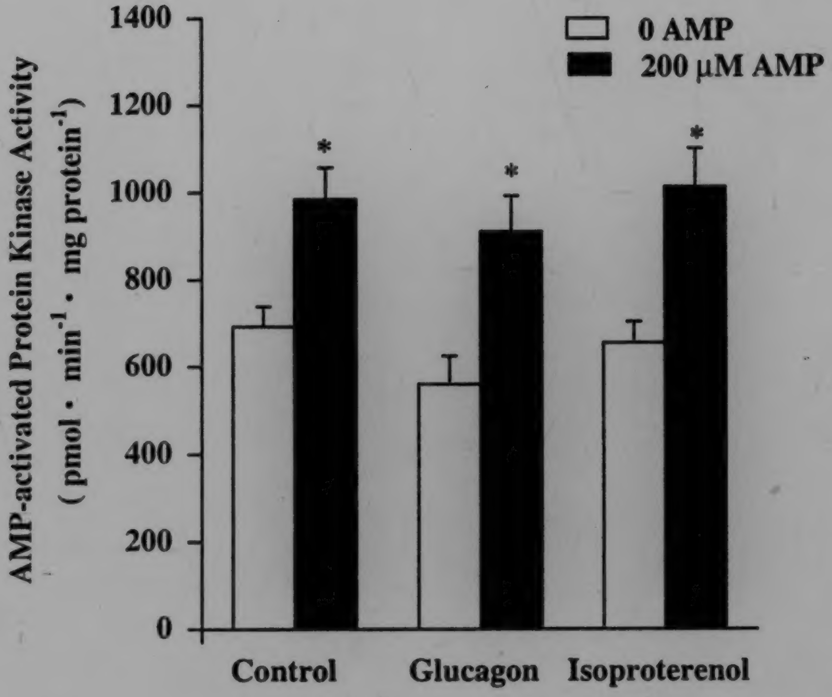

AMP-activated Protein Kinase Activity
(pmol • min^{-1} • mg protein^{-1})
1400
1200
1000
800
600
400
200
0
☐ 0 AMP
■ 200 μM AMP
*
*
*
Control
Glucagon
Isoproterenol

Figure 7-3:

ACC activity measured in 6% polyethylene fractions obtained from 7-day old rabbit hearts perfused with glucagon or isoproterenol.

ACC activity was determined by the $^{14}CO_2$ fixation assay as described in Methods. Values shown are the mean ± SEM of results from 8 control hearts and 6 hearts each for glucagon and isoproterenol-treated groups.
* significantly different from control hearts.

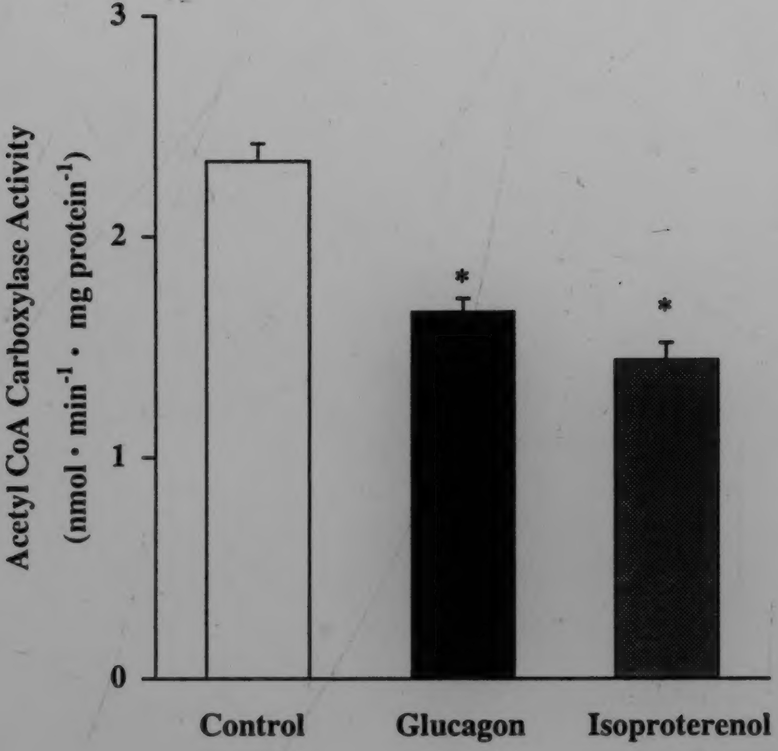

Acetyl CoA Carboxylase Activity
(nmol · min^{-1} · mg protein^{-1})
3
2
1
0
*
*
Control
Glucagon
Isoproterenol

Figure 7-4:

Malonyl CoA levels determined in 6% perchloric acid extracts obtained from 7-day rabbit hearts perfused with glucagon or isoproterenol.

Malonyl CoA levels were determined as described in Methods.
Values shown are the mean ± SEM of results from 8 control hearts and 6 hearts each for glucagon and isoproterenol-treated groups.
* significantly different from control hearts

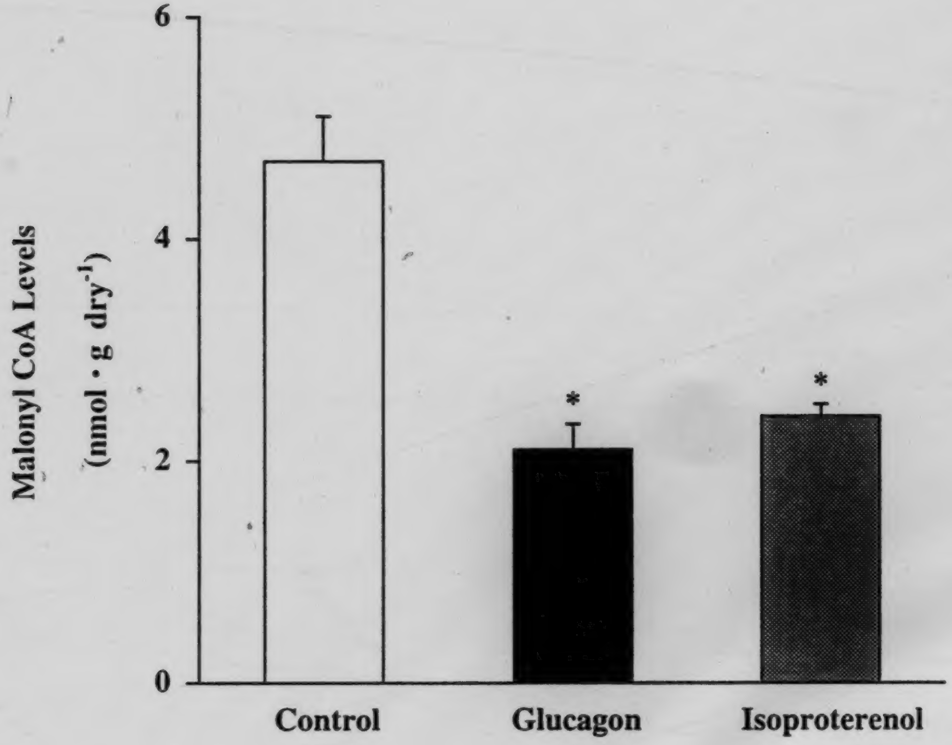

Malonyl CoA Levels
(nmol · g dry^{-1})
6
4
2
0
*
*
Control
Glucagon
Isoproterenol

Figure 7-5:

Representative immunoblot analysis of 6% PEG 8000 extracts obtained from 7-day old rabbit hearts perfused with glucagon.

Immunoblot analysis was performed as described in Methods. (A) AMPK α1 or α2 catalytic subunit (B) ACC protein. Neither AMPK nor ACC protein abundance was altered by glucagon.

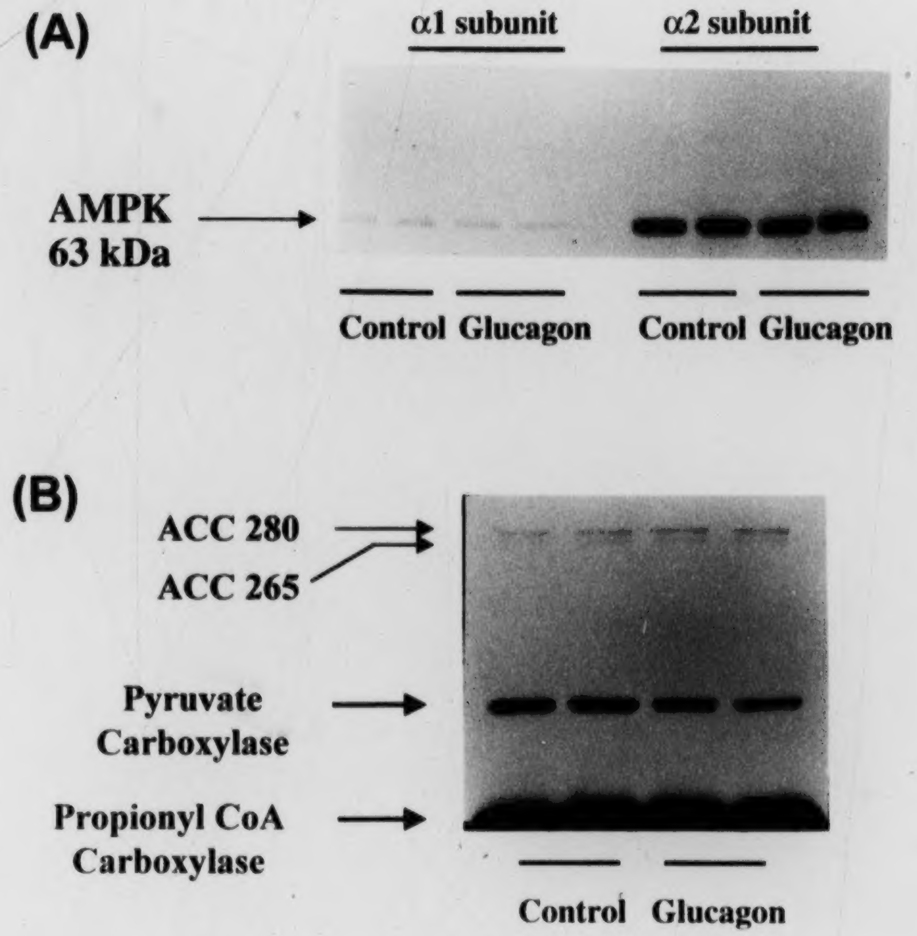

(A)
α1 subunit
α2 subunit
AMPK
63 kDa
Control
Glucagon
Control
Glucagon
(B)
ACC 280
ACC 265
Pyruvate
Carboxylase
Propionyl CoA
Carboxylase
Control
Glucagon

Figure 7-6:

cAMP levels determined in 10% trichloroacetic acid extract obtained from 7-day rabbit hearts perfused with glucagon or isoproterenol.

cAMP levels were determined as in Methods.

Values shown are the mean ± SEM of results from 8 control hearts and 6 hearts each for glucagon and isoproterenol-treated groups.

* significantly different from control heart.

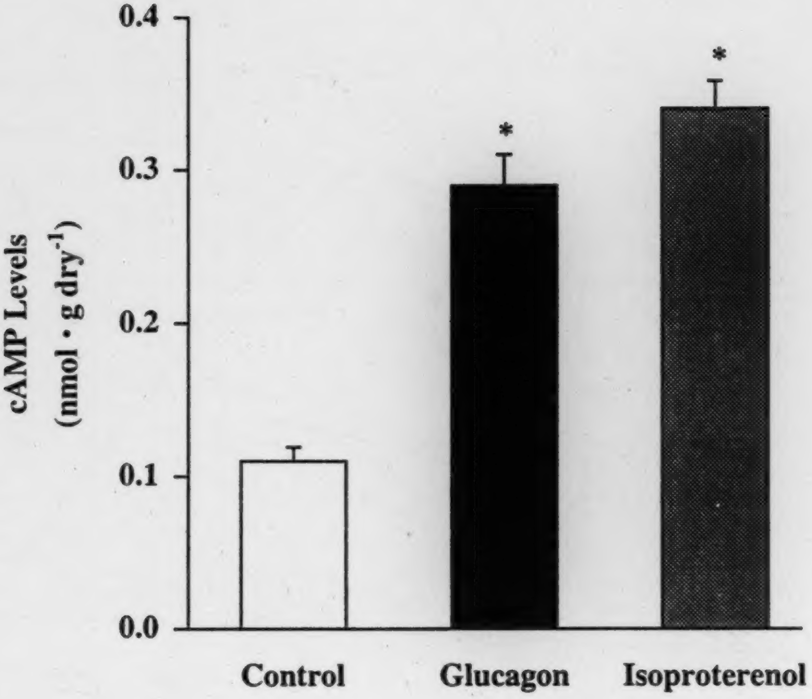
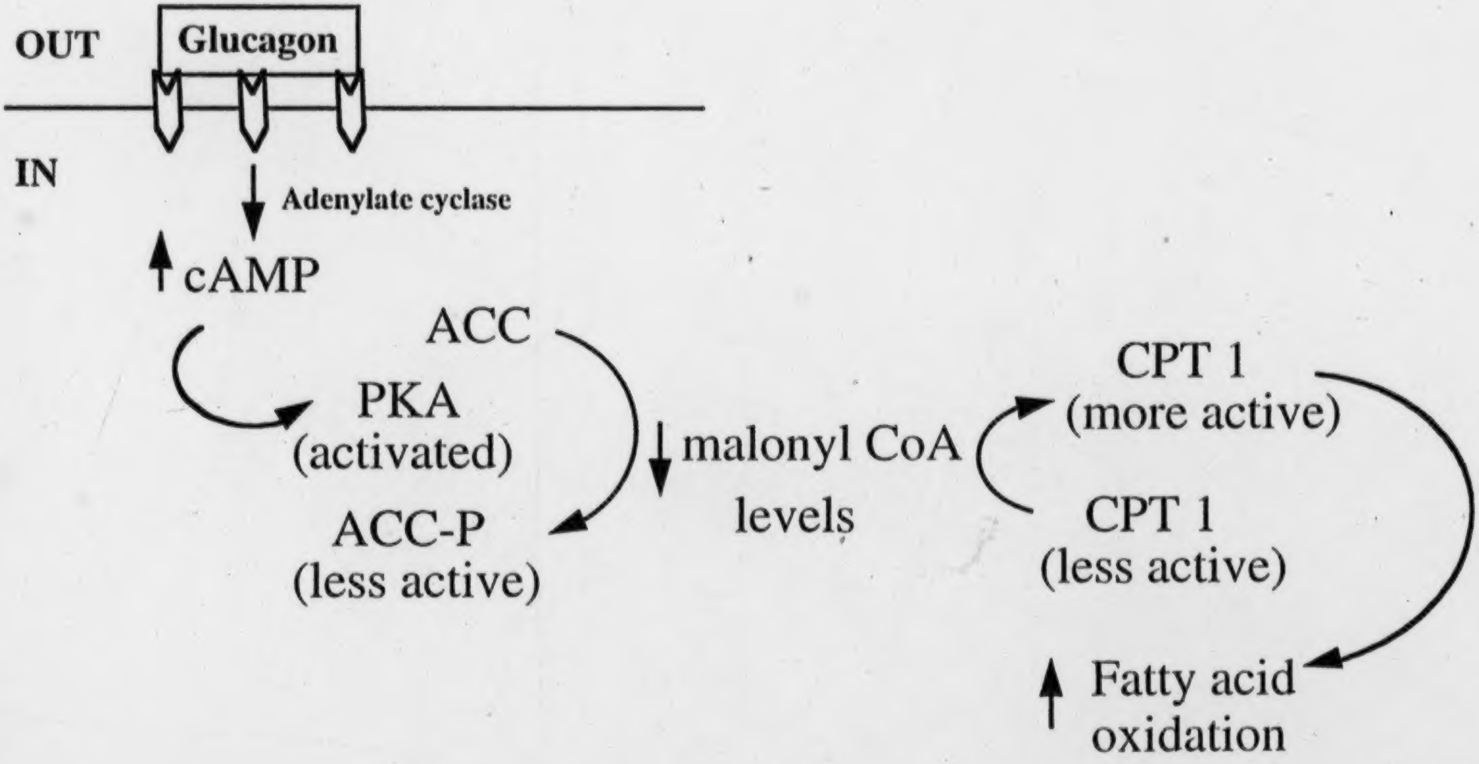

0.4
0.3
0.2
0.1
0.0
cAMP Levels
(nmol · g dry⁻¹)
*
*
Control
Glucagon
Isoproterenol

Figure 7-7:

Proposed scheme showing pathway by which an increase in glucagon levels may contribute to increased fatty acid oxidation rates in the newborn heart.

The increased glucagon level in blood following birth results in increased cAMP levels. This leads to an activation of cAMP dependent protein kinase (PKA). The result of PKA activation is an increased phosphorylation and inactivation of ACC, resulting in a decrease in myocardial malonyl CoA levels. This relieves the inhibition of carnitine palmitoyltransferase 1 (CPT 1), the rate limiting enzyme involved in mitochondria uptake of activated fatty acids. The ultimate result is an increase in fatty acid oxidation.


OUT
Glucagon
IN
Adenylate cyclase
↑ cAMP
ACC
PKA
(activated)
ACC-P
(less active)
↓ malonyl CoA
levels
CPT 1
(more active)
CPT 1
(less active)
↑ Fatty acid
oxidation

References:

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Chapter 8

General Discussion and Conclusions

General Discussion

Earlier studies in our laboratory demonstrated that the rate of fatty acid oxidation increased dramatically in 7-day compared to 1-day old rabbit hearts (Lopaschuk and Spafford, 1990). This increase in fatty acid oxidation results from both a decrease in ACC activity and a concomitant decrease in levels of malonyl CoA (Lopaschuk et al., 1994b) in 7-day old hearts. At the beginning of the studies presented in this thesis, the mechanism responsible for the decrease in ACC activity in the 7-day old heart was unknown. Previous studies in rat liver, however, showed that ACC could be phosphorylated and inactivated by a novel AMPK (Davies et al., 1990; Davies et al., 1992). A possible candidate kinase responsible for the decreased ACC activity in 7-day old hearts therefore appears to be AMPK. However, whether or not AMPK is actually expressed or active in the newborn rabbit heart (1-day or 7-day old) was unknown at the beginning of my studies. I hypothesized, that an increase in AMPK protein abundance and/or activity in 7-day compared to 1-day old hearts is primarily responsible for the decrease in ACC activity and the dramatic increase in fatty acid oxidation in 7-day old hearts. To test this hypothesis, I determined whether or not AMPK was actually expressed in newborn rabbit hearts.

Identification of AMPK in both fetal and newborn rabbit hearts:

Results presented in Chapter 3 of this thesis directly demonstrate that newborn rabbit hearts expresses an active 5'AMP-activated protein kinase. As can be seen from Figures 3-2A & B and Figures 3-4A & B, 7-day old hearts express α, β, and γ subunits of AMPK. This result is consistent with previous findings by Woods et al. (1996a; 1996b), which characterized α, β, and γ subunits of AMPK in the rat liver. Although the nature of AMPK subunit association in the newborn rabbit heart still remains to be determined, it is possible that they are associated with each other in a heterotrimeric complex in a fashion similar to rat liver AMPK subunit association.

Another finding from this study is that AMPK can be detected prior to birth in fetal rabbit hearts (Figure 3-6A). Moreover, Figure 3-6B demonstrates that ACC protein can be detected in fetal rabbit hearts. In fact ACC 280 kDa appears to be the only ACC isoform present in the fetal rabbit heart as opposed to the presence of both ACC 280 and ACC 265 kDa isoforms which are detected in the 1-day old heart. At present, our knowledge of the role of AMPK in regulating energy metabolism in the fetal heart is still unclear. Recent studies in the literature, however, suggest that AMPK may be involved in regulating glucose metabolism (Young et al., 1996). Since the fetal heart is more dependent on glycolysis (see Rolph and Jones, 1985 for review) and there exists a measurable AMPK activity in these hearts (Figure 3-7), it is possible that fetal heart AMPK may be involved in regulating carbohydrate metabolism, but this remains to be determined.

Table 3-2 shows that in newborn hearts, AMPK activity is high in our *in-vitro* assay using synthetic peptide substrates. Indeed the highest activity was recovered in 6% PEG 8000 extracts. This activity is comparable with that measured by Kudo et al. (1996) and Gamble and Lopaschuk (1997), who measured AMPK activity in adult rat hearts. It is of interest that basal AMPK activity could be stimulated in the presence of 200 μM AMP (Figures 3-8, 3-9 & 3-10). This AMP activation of AMPK should not be confused with cAMP activation of protein kinase A (PKA). Increased AMP levels have been suggested to allosterically activate AMPK. AMP may also act via an activation of an upstream AMPK kinase (AMPKK). This AMPKK has been recently identified and characterized in the rat liver, and threonine 172 has been identified as the major site on AMPK which is phosphorylated by AMPKK (Weekes et al., 1994; Hawley et al., 1996). As discussed below, indirect evidence suggests that AMPKK is present in the newborn heart, although the identification and characterization of AMPKK in newborn hearts has yet to be fully elucidated.

Phosphorylation control of the newborn heart AMPK:

Since the newborn heart expresses an active AMPK, we examined the potential mechanism by which this AMPK is regulated. Phosphorylation of rat liver AMPK results in an increased AMPK activity (Moore et al., 1991; Davies et al., 1995). To gain an insight into the phosphorylation control of newborn heart AMPK, 6% PEG 8000 extracts were obtained in the presence or absence of sodium fluoride and sodium pyrophosphate (protein phosphatase inhibitors). Indeed as shown in Figure 3-11, extraction of newborn heart AMPK in the absence of sodium fluoride and sodium pyrophosphate resulted in a significantly decreased AMPK activity when compared to extracts obtained in the presence of sodium fluoride and sodium pyrophosphate. This finding is further supported by results obtained following incubation of 6% PEG 8000 extracts with ATP/Mg^{2+} or with either PP2A or PP1C (both protein phosphatase inhibitors). As shown in Figures 3-13 and 3-14 incubation with ATP/Mg^{2+} resulted in a significant increase in AMPK activity, whereas incubation with PP2A or PP1C significantly decreased AMPK activity compared to control incubations. These data are consistent with AMPK being active in its phosphorylated state (Moore et al., 1991). It would appear that changes in the phosphorylation status of newborn heart AMPK can result in changes in AMPK activity.

Regulation of ACC by AMPK in the newborn rabbit heart:

Simultaneous measurement of AMPK and ACC activity in the same 6% PEG 8000 extracts (Chapter 3) demonstrates that higher AMPK activity in these extracts is obtained in the presence of sodium fluoride and sodium pyrophosphate is associated with lower ACC activity. These results provide indirect evidence that increased AMPK activity may result in increased phosphorylation and inactivation of ACC in the newborn rabbit heart. I propose that increased AMPK activity results in an increase in phosphorylation and inactivation of ACC. This results in a decreased malonyl CoA level that relieves its inhibition of CPT 1 activity. The resultant increased activity of CPT 1 increases fatty acid oxidation through the β-oxidation spiral within the mitochondria (see Figure 3-15).

Overall I have shown that AMPK is expressed in the newborn rabbit heart with measurable activity. This AMPK is also expressed prior to birth in fetal rabbit hearts. Moreover, I have demonstrated that AMPK activity is increased by phosphorylation and can be decreased by dephosphorylation in an assay. Furthermore, I have provided evidence that increased AMPK activity is associated with decreased ACC activity. It is possible that the decrease in ACC activity observed in 7-day old rabbit hearts compared to 1-day old hearts (Lopaschuk et al., 1994b) results directly from an increase in AMPK activity. I therefore investigated whether increased AMPK activity and/or protein abundance could explain the decrease in ACC activity and the rapid maturation of fatty acid oxidation in the 7-day old heart.

The role of AMPK in the increased myocardial fatty acid oxidation in the immediate newborn period:

Having demonstrated that AMPK is present in the newborn heart and that it may regulate ACC activity, I determined if differences in AMPK abundance and/or activity between 1-day and 7-day old hearts could explain the decreased ACC activity and increased fatty acid oxidation seen in 7-day old hearts. Indeed AMPK activity was increased significantly in 7-day old hearts compared to 1-day old hearts (Figure 4-1). Moreover, AMPK immunoreactive protein abundance increased significantly in 7-day compared to 1-day old hearts.

The reason for the increased AMPK immunoreactive protein abundance in 7-day old compared to 1-day old hearts is not clear. It is possible that activation of the promoter region of AMPK results in increased AMPK mRNA that results in an increase in AMPK immunoreactive protein, but this remains speculative. Increased AMPK immunoreactive protein abundance could also be accounted for by an increase in stability or a decreased degradation of AMPK in the 7-day old heart. Further studies to confirm these possibilities are necessary. It is, however, conceivable, that upregulation of AMPK immunoreactive protein and activity in the 7-day old heart plays a role in the decreased ACC activity and increased myocardial fatty acid oxidation post-birth. Previously, we demonstrated that ACC protein levels do not change between 1-day and 7-days post birth in the rabbit heart (Lopaschuk et al., 1994b), a finding confirmed by my studies. ACC activity and malonyl CoA levels, however, decrease precipitously in 7-day compared to 1-day old hearts (Lopaschuk et al., 1994b). Since AMPK can phosphorylate and inactivate ACC in the rat heart (Davies et al., 1992) and AMPK protein and activity increased in 7-day compared to 1-day old hearts, increased AMPK activity may be directly responsible for the decrease in ACC activity in the 7-day old heart. I, therefore, determined whether changes in ACC activity are associated with changes in the rate of fatty acid oxidation.

To investigate this possibility, hearts from 1-day and 7-day old rabbits were perfused with or without insulin (100 μU/ml) and the rate of fatty acid oxidation, ACC activity and AMPK activity were measured directly. As can be seen from Figure 4-3, the rate of fatty acid oxidation in the 7-day old heart was significantly increased compared to 1-day old hearts. In addition, insulin decreased the rate of fatty acid oxidation in 1-day old hearts. This insulin-induced decrease in the rate of fatty acid oxidation in 1-day old hearts was accompanied by a significant increase in ACC activity (see Figures 4-3 & 4-4) compared to 7-day old hearts. Interestingly, AMPK activity in hearts perfused with insulin was significantly decreased compared to control hearts (Figure 4-5). This observation is consistent with previous findings by Witters and Kemp (1992) who demonstrated that insulin stimulates ACC activity in isolated rat hepatocytes secondary to an inhibition of AMPK activity.

Since circulating blood insulin levels decrease in the immediate newborn period (Girard et al., 1992), I suggest that a decrease in insulin levels combined with the increased AMPK abundance in the 7-day old rabbit heart results in an increase in AMPK activity and a decrease in ACC activity. This provides a plausible explanation of the mechanism responsible for decreased ACC activity in 7-day old hearts previously reported by Lopaschuk et al. (1994b). My results also explain the rapid decline in levels of malonyl CoA secondary to decreased ACC activity in 7-day compared to 1-day old rabbit hearts.

To date, the mechanism by which high insulin levels decrease AMPK activity is unclear. Insulin had been reported to stimulate protein phosphatase 1 activity in rat adipocytes (Begum, 1995). In my studies, protein phosphatase 2A and 1C can inhibit AMPK activity in newborn hearts (see Chapter 3; Figure 4-6).

It is, therefore, possible that insulin may stimulate protein phosphatase 2A or 1C in the newborn heart, resulting in dephosphorylation and inactivation of AMPK. Another possibility is that insulin may directly interact with the catalytic or regulatory subunit of AMPK, resulting in decreased AMPK activity. These possibilities are speculative and are open to further investigation.

Taken together, my data demonstrate that increased AMPK activity (arising from both an increase in abundance and decrease in circulating insulin levels) results in decreased ACC activity in 7-day compared to 1-day old hearts. This decrease in ACC activity is accompanied by a decrease in malonyl CoA levels (Lopaschuk et al., 1994b). The ultimate result is an increase in CPT 1 activity and a concomitant increase in β-oxidation of fatty acids within the mitochondria in 7-day old hearts (see Figure 4-7).

Evidence that AMPK regulates fatty acid oxidation in the newborn rabbit heart:

Although I demonstrated that AMPK is upregulated in the 7-day old heart and is accompanied by a decrease in ACC activity and an increase in fatty acid oxidation compared to 1-day old hearts, a direct cause and effect relationship between AMPK and fatty acid oxidation has yet to be clearly established. Using AICAR, a cell permeable nucleoside analogue, I demonstrated that AICAR could directly stimulate AMPK activity and the rate of fatty acid oxidation in 7-day old hearts (Figures 5-1 and 5-2).

AICAR is rapidly converted to ZMP within the cell. ZMP then acts as an AMP analogue to directly activate AMPK (Corton et al., 1995). This stimulation of AMPK activity by AICAR is consistent with a previous observation in rat adipocytes (Sullivan et al., 1994) and recent studies in rat hepatocytes and skeletal muscle (Merrill et al., 1997; Samari and Seglen, 1998; Hayassi et al., 1998) which also demonstrated that AICAR stimulates AMPK activity. In addition, the increase in the rate of fatty acid oxidation with AICAR was indeed associated with an increase in AMPK activity (Figure 5-2). When iodotubercidin, an AMPK inhibitor, was used to perfuse 7-day old hearts, AMPK activity was significantly decreased which was accompanied by a significant decrease in the rate of fatty acid oxidation compared to controls (Figures 5-1 & 5-2). As would also be expected, ACC activity decreased or increased, respectively, in AICAR or iodotubercidin-treated hearts (Figure 5-3). It is, however, important to note that neither AICAR nor iodotubercidin significantly altered cardiac work performed by the heart (Table 5-1).

Since iodotubercidin is not a specific AMPK inhibitor, I also used another AMPK inhibitor, 2'deoxyadenosine, in perfused 7-day old hearts. As shown in Table 5-3 2'deoxyadenosine also significantly decreased AMPK activity, increased ACC activity and caused a significant decrease in the rate of fatty acid oxidation. These results demonstrate that in the 7-day old heart, AMPK can be pharmacologically modulated. In addition, these results show that stimulation or inhibition of AMPK, respectively, can directly result in increased or decreased rate of fatty acid oxidation. Henin et al. (1996) previously showed that iodotubercidin can inhibit AMPK in rat hepatocytes, my results with iodotubercidin in this study are in agreement with their findings. My results are also consistent with recent findings by Merrill et al. (1997) which demonstrate that AICAR by stimulating AMPK activity in skeletal muscle results in increased rates of fatty acid oxidation. Under conditions of increased fatty acid oxidation, levels of malonyl CoA would be expected to decrease. In this study, the level of malonyl CoA was significantly decreased in AICAR-treated hearts and was increased in iodotubercidin-treated hearts compared to controls (Figure 5-4). This is consistent with an increased or decreased ACC activity, respectively, by iodotubercidin or AICAR. Levels of malonyl CoA reported in this study, however, are similar to previously measured values in newborn rabbit hearts (Lopaschuk et al. 1994b).

Earlier studies in our laboratory and others demonstrate that heart CPT 1 activity is very sensitive to malonyl CoA inhibition (Saddik et al., 1993; Kudo et al., 1995; see McGarry and Brown, 1997 for review). Decreased levels of malonyl CoA could, therefore, be expected to result in an increase in CPT 1 activity with resultant increase in fatty acid oxidation. My data are, therefore, consistent with a decrease in levels of malonyl CoA resulting in increased CPT 1 activity and an acceleration of fatty acid oxidation. Interestingly, neither changes in ACC activity nor changes to AMPK activity were due to alterations in ACC or AMPK protein abundance (Figures 5-5A & 5-5B). As shown in Figures 5-6 and 5-7, increased AMPK activity was negatively correlated with a decrease in ACC activity. Moreover, increased AMPK activity was positively correlated with increased fatty acid oxidation. These results, therefore, demonstrate that an increase in AMPK activity results in decreased ACC activity and that activation of AMPK activity results in increased fatty acid oxidation in newborn heart.

In summary, I have shown that newborn heart AMPK can be pharmacologically stimulated or inhibited, respectively, by AICAR or iodotubercidin (and 2'deoxyadenosine). Increase in AMPK activity is associated with an increase in the rate of fatty acid oxidation. Alternatively, a decrease in AMPK activity is associated with decreased rates of fatty acid oxidation. These data, therefore, provide evidence that AMPK is an important regulator of the rate of fatty acid oxidation in the newborn heart.

AMPK modulation of fatty acid oxidation and recovery of heart function in the reperfused-ischemic newborn rabbit heart:

My successful demonstration of increased or decreased rates of fatty acid oxidation with AICAR or iodotubercidin, respectively, led us to investigate the effects of AMPK modulators on the recovery of heart function in newborn hearts subjected to a transient period of global no flow ischemia. High levels of plasma fatty acid have previously been reported in infants and adults following myocardial ischemia (Lopaschuk et al., 1994a). High rates of fatty acid oxidation in the reperfused-ischemic hearts are, however, associated with an increase in AMPK activity (Kudo et al., 1995) and depressed functional recovery. I therefore hypothesized that inhibition of AMPK activity results in a decreased rate of fatty acid oxidation and an improved recovery of heart function. Conversely, an increase in AMPK activity results in increased rates of fatty acid oxidation and a further depression of functional recovery in reperfused–ischemic hearts.

To test this hypothesis, 7-day old hearts were subjected to ischemia-reperfusion protocol as shown in Figure 6-1. In the absence of any AMPK modulator, cardiac function recovered to approximately 52% of pre-ischemic values (Table 6-1). This value is comparable to previous findings by Itoi et al. (1993). It is of interest, however, that ischemia resulted in an increase in AMPK activity which was accompanied by a decrease in ACC activity at the end of ischemia (Figures 6-2 & 6-3). Neither the change in AMPK nor ACC activity was due to ischemia-induced alteration in AMPK or ACC protein abundance (Figure 6-4). If, however, AICAR or iodotubercidin was included in the perfusion buffers, the increase or decrease in AMPK activity, respectively, was accompanied by a decrease or increase in ACC activity in the reperfused-ischemic hearts (Figures 6-5 and 6-6). Rates of fatty acid oxidation in reperfused-ischemic hearts also increased or decreased, respectively, in AICAR or iodotubercidin-treated hearts (Figure 6-7). Another AMPK inhibitor, 2'deoxyadenosine also decreased the rate of fatty acid oxidation compared to controls (Figure 6-7) during reperfusion of previously ischemic hearts suggesting that inhibition of AMPK results in a decrease in the rate of fatty acid oxidation. Since high rates of fatty acid oxidation are associated with depressed functional recovery, inhibition of fatty acid oxidation would be expected to improve functional recovery in reperfused-ischemic hearts. Indeed both iodotubercidin and 2'deoxyadenosine significantly improved the recovery of mechanical function in newborn hearts subjected to an ischemia–reperfusion protocol (Figure 6-8). Moreover, both agents also decreased AMPK activity and increased ACC activity in 7-day old hearts. This observation was recently confirmed in our laboratory in rat hearts where iodotubercidin improved recovery of mechanical function in adult rat hearts subjected to an ischemia-reperfusion protocol (Dr. Taniguchi Masayuki, personal communication).

The reason why high rates of fatty acid oxidation are detrimental to the recovery of mechanical function in the reperfused ischemic heart is still not completely understood. An imbalance between glycolysis and glucose oxidation, resulting in severe acidosis has been proposed as a possible explanation (Lopaschuk et al., 1992). This hypothesis was recently supported by Liu et al. (1996) who demonstrated that although TCA cycle activity recovers following ischemia-reperfusion in rat hearts, glucose oxidation was depressed compared to pre-ischemic values. Moreover, TCA cycle activity and ATP production were uncoupled from contractile activity of the cardiomyocytes as evidenced by the poor functional recovery. Since dichloroacetate improved heart recovery in their study by stimulating glucose oxidation, it is conceivable that stimulation of glucose oxidation decreases fatty acid oxidation in dichloroacetate-treated hearts resulting in an enhanced functional recovery. Increased glucose oxidation or decreased fatty acid oxidation, therefore, has the potential to improve heart function following ischemia. In my studies, iodotubercidin and 2'deoxyadenosine inhibited AMPK activity, decreased rates of fatty acid oxidation and improved the recovery of heart function following ischemia. Although the mechanism by which these agents exert their effect on AMPK is unclear, the possibility exists that they bind to an allosteric site on AMPK, thereby preventing the activation of AMPK. If AMPK inhibitors significantly improve the recovery of heart function in the clinical setting as it does in these pre-clinical experiments, inhibition of AMPK may be a novel approach to treating ischemic heart disease.

The contribution of glucagon to the increase in myocardial fatty acid oxidation rates in the newborn rabbit heart:

As discussed, AMPK is important in the maturation of fatty acid oxidation in newborn heart. AMPK activity, however, can be stimulated by glucagon in isolated rat hepatocytes (Sim and Hardie, 1988). Interestingly, circulating glucagon levels rise during the same period that fatty acid oxidation increases in newborn heart (Girard et al., 1992). I therefore hypothesized that glucagon stimulates the rate of fatty acid oxidation by decreasing ACC activity secondary to an increase in AMPK activity. In testing this hypothesis, my main objective was to determine if glucagon could increase the rate of fatty acid oxidation and activate AMPK activity in the newborn heart. To achieve this, 7-day rabbit hearts were perfused in the presence or absence of glucagon. As shown in Figure 7-1, glucagon significantly increased the rate of fatty acid oxidation. This increase in the rate of fatty acid oxidation, however, was not associated with an increase in AMPK activity (Figure 7-2). Moreover, neither α1 nor α2 catalytic subunit of

AMPK was significantly altered by glucagon (Figure 7-5A). Interestingly, a significant decrease in ACC activity and levels of malonyl CoA were observed (Figures 7-3 & 7-4). ACC protein content, however, was not significantly altered (Figure 7-5B). It would appear therefore that glucagon stimulated rate of fatty acid oxidation and decreased ACC activity via an AMPK independent mechanism. This result is consistent with previous findings by Hall et al. (1996) which demonstrates that dobutamine-induced increase in fatty acid uptake was not accompanied by changes in AMPK activity.

One possible reason for the decrease in ACC activity in glucagon perfused hearts may be related to an increase in levels of cAMP that result in activation of protein kinase A (PKA). PKA can phosphorylate and inactivate ACC in rat adipocytes (Haystead et al., 1990). Moreover, previous studies in isolated hepatocytes showed that glucagon can induce phosphorylation and inactivation of ACC by a cAMP dependent protein kinase mechanism (Holland et al., 1984). It is, therefore, possible that glucagon activates PKA by increasing levels of cAMP with resultant phosphorylation and inactivation of ACC. To confirm this possibility, hearts were perfused with isoproterenol, another agent known to increase levels of cAMP and PKA activity. As shown in Table 7-1, both isoproterenol and glucagon significantly increased cardiac work compared to control hearts. Interestingly, the effects of isoproterenol on the rate of fatty acid oxidation, AMPK activity, and ACC activity were very similar to those previously observed with glucagon (see Figures 7-1, 7-2 & 7-3). It therefore appears that like glucagon, isoproterenol also increases rate of fatty acid oxidation and decreases ACC activity via an AMPK independent mechanism. It is of interest that levels of malonyl CoA decreased significantly in parallel with decreased ACC activity in both glucagon and isoproterenol-treated hearts compared to controls (Figure 7-4).

To further elucidate the mechanism by which glucagon and isoproterenol decreased ACC activity in the heart, we measured levels of cAMP in both glucagon and isoproterenol-treated hearts. As expected, levels of cAMP in both groups increased significantly (see Figure 7-6) compared to control hearts. These results suggest that both glucagon and isoproterenol may stimulate PKA activity secondary to increased levels of cAMP. It also suggests that decreased ACC activity may have resulted from a PKA induced phosphorylation and inactivation of ACC. Increased AMP/ATP ratio has been recently suggested to be a more sensitive indicator of activation of AMPK during cellular stress response (Corton et al., 1994). As shown in Table 7-2 however, neither glucagon nor isoproterenol significantly altered this ratio. Moreover, the levels of AMP or ATP were not significantly altered. These results further confirm that both glucagon and isoproterenol decreased ACC activity by an AMPK independent mechanism.

Isoproterenol stimulation of fatty acid oxidation and implication for inotropes as adjuvants in pediatric cardiac surgery:

The serendipitous finding that isoproterenol (a β-agonist) increases the rate of fatty acid oxidation in newborn rabbit hearts (Figure 7-1) is contrary to previous findings in the adult rat heart where β-agonists selectively stimulate glucose oxidation (Collins-Nakai et al., 1994). Since high rates of fatty acid oxidation are detrimental to reperfusion recovery due to their inhibitory effect on glucose metabolism (Kudo et al., 1995), it is conceivable that isoproterenol may worsen the recovery of mechanical function post ischemia in newborn hearts. This possibility is presently being investigated in our laboratory. However, it is possible that the routine use of β-agonists to improve heart function in pediatric cardiac surgery may not confer an extra advantage for an excellent functional recovery post-surgery due at least in part to an excessive stimulation of rate of fatty acid oxidation.

Maturation of fatty acid oxidation in the newborn rabbit heart:

Combined together, results of studies presented in this thesis suggest that a rise in glucagon levels post-birth coupled with the rapid decline in insulin (Girard et al., 1992) may act in concert to contribute to the rapid maturation of fatty acid oxidation in the newborn heart. As proposed in Figure 4-7, a decrease in insulin levels results in an increased rate of fatty acid oxidation secondary to an increase in AMPK activity. Moreover, a rise in glucagon levels results in an increased rate of fatty acid oxidation secondary to increased levels of cAMP and activation of PKA. A schematic diagram depicting this possibility is shown in Figure 8-1.

Significance of this study:

Overall, results presented in this thesis demonstrate that AMPK is an important regulator of myocardial fatty acid oxidation in the newborn heart. Since AMPK is highly expressed in the heart and pharmacological modulation of AMPK activity alters the rate of fatty acid oxidation in newborn heart, it should be possible to develop newer AMPK modulators which can stimulate or inhibit fatty acid oxidation in the heart. This may involve a collaborative effort between the pharmacologist and medicinal chemist.

Moreover, a potential new class of agents which can improve the recovery of cardiac function has been identified from results presented in this thesis. AMPK inhibitors may be a new class of agents useful in the treatment of ischemic heart disease. In addition if AMPK inhibitors can also decrease the rate of fatty acid oxidation in the diabetic heart (where over-reliance on fatty acid oxidation has been associated with diabetic cardiomyopathies Lopaschuk et al. 1994c) AMPK inhibitors may offer new hopes for patients suffering from diabetes induced cardiomyopathies.

Limitations of this study:

Whereas studies presented in this thesis are facilitated by the availability of a perfusion system that allows us to directly measure the rate of fatty acid oxidation while directly measuring changes in AMPK and ACC activity, the perfusion system is by no means an ideal or perfect system and there are some limitations to the possible interpretations of results presented in this thesis. These limitations are discussed below.

1) All isolated heart perfusions described in this thesis involve removing the heart from newborn rabbits and perfusing them in an *ex vivo* condition with relevant energy substrates. Therefore, this is not a whole animal study and blood (and blood factors) are absent from our perfusion system. As such, the absence of hemoglobin means a poor oxygen carrying capacity of our re-circulating perfusate. Moreover, absence of leukocytes and monocytes may subject perfused hearts to decreased resistance to immune challenge by cytokines.
2) The perfusion system also utilizes a situation in which the newborn hearts were perfused with 95% O_2 / 5% CO_2 gaseous mixture. This results in a higher oxygen tension to which hearts are exposed to compared to within the intact animal. Albeit, it is under this condition that we can sustain heart perfusion *ex vivo* due to limitations already discussed above.

3) ACC protein content was not significantly altered while its activity changed under certain conditions in some studies presented in this thesis. Changes in ACC activity, therefore, may be due to phosphorylation/dephosphorylation control of ACC activity. One technical limitation encountered during my studies is that it is not possible to directly quantify the amount of phosphate incorporated into ACC under different experimental conditions described in this thesis. Availability of phosphopeptide analysis methodology to investigate differences in phosphate incorporation into ACC in control, iodotubercidin, 2'deoxyadenosine and AICAR treated hearts would further strengthen the validity of results presented in this thesis.

4) Levels of malonyl CoA measured in this study are total tissue malonyl CoA levels. We do not yet have the technical ability to specifically measure cytosolic and mitochondrial malonyl CoA levels separately. To our knowledge, no method is currently available for differential measurement of malonyl CoA levels in cellular sub-compartments. Development of such methodology will no doubt enhance our understanding of malonyl CoA regulation of fatty acid oxidation in the heart. Malonyl CoA has been postulated to be sub-compartmentalized in the rat liver (Guzman and Geelen, 1992). Whether or not this sub-compartmentalization exists in the newborn rabbit heart is unknown.

5) Perfusions with different agents described in this thesis were carried out on an acute basis. The effect of prolonged administration of these agents remains unknown.

6) The dose response curve has always been the domain of the pharmacologist. Owing to limitations and the highly expensive nature of most of the newer test drugs (iodotubercidin, 2'deoxyadenosine) used in this study, dose dependence of effects of agents could not be carried out in these studies. It

would be interesting to see what higher or lower doses of AMPK modulators do to AMPK and ACC activities and the rate of fatty acid oxidation in newborn hearts.

Summary:

Listed below is a brief summary of major findings from this thesis.

1. Newborn rabbit heart contains an α catalytic subunit and β and γ regulatory subunits of AMPK. AMPK is present prior to birth at 29-days of gestation (term is usually 31 days) in the fetal rabbit heart.
2. Both $\alpha 1$ and $\alpha 2$ catalytic subunits of AMPK are present in the newborn rabbit heart.
3. The newborn rabbit heart AMPK is subject to phosphorylation /dephosphorylation control by kinases and phosphatases respectively. Increased AMPK activity is associated with decreased ACC activity.
4. AMPK catalytic subunit abundance was greater in the 7-day old heart compared to 1-day old rabbit hearts. This increased AMPK abundance was associated with decreased ACC activity.
5. High insulin levels significantly decrease AMPK activity and increase ACC activity. Hence, the rapid decrease in insulin levels following birth results in increased AMPK activity and decreased ACC activity.
6. Glucagon increases the rate of fatty acid oxidation without significant changes to AMPK protein or the activity and may activate PKA secondary to an increase in the levels of cAMP in the newborn heart.
7. Isoproterenol increases the rate of fatty acid oxidation in newborn rabbit hearts by an AMPK independent mechanism. This effect is different from the

previously reported effect of isoproterenol in the adult rat heart where isoproterenol stimulated glucose oxidation.

8. AICAR increases AMPK activity and decreases ACC activity in the newborn rabbit heart, whereas iodotubercidin decreases AMPK activity and increases ACC activity.
9. A significant negative correlation exists between AMPK activity and ACC activity in the newborn rabbit heart. More importantly, an increase in AMPK activity is positively correlated with an increase in the rate of fatty acid oxidation.
10. An ischemic episode of 40 min results in a five fold increase in cellular AMP levels compared to control aerobic hearts and is accompanied by a stimulation of AMPK activity.
11. The AMPK activator AICAR depresses functional recovery of newborn hearts during reperfusion following ischemia. In contrast the AMPK inhibitor iodotubercidin improves functional recovery in newborn hearts reperfused following ischemia.
12. The decrease in insulin levels and an increase in glucagon levels following birth are associated with a decrease in ACC activity and an increase in the rate of fatty acid oxidation following birth in newborn rabbit heart.

Data presented in this thesis have improved our understanding of the possible mechanism responsible for the increase in fatty acid oxidation in 7-day compared to 1-day old hearts. In particular, these results demonstrate that decreased in ACC activity observed in 7-day old hearts is due to increased AMPK activity.

Moreover, the decrease in malonyl CoA levels observed in 7-day old heart probably results from the decrease in ACC activity (the product of heart ACC activity). This may result in a significant increase in CPT 1 activity since malonyl CoA is a potent inhibitor of the CPT 1 activity. The ultimate result is an increase in the translocation of activated fatty acid into the mitochondria for eventual β-oxidation. Furthermore, the observation that AMPK activator, AICAR decreases functional recovery in reperfused-ischemic heart and that AMPK inhibitors improve recovery of mechanical function during reperfusion following ischemia strongly suggests that, changes in AMPK-ACC regulatory system (which alters myocardial fatty acid oxidation) is important in recovery of heart function post-ischemia.

Since plasma fatty acid levels increase in infant and adult patients following acute myocardial infarction, (Lopaschuk et al., 1994a) these hearts may be overly reliant on fatty acid oxidation. It is possible that AMPK inhibitors may lower excessive reliance of these hearts on fatty acid oxidation. In that instance, AMPK inhibitors may be promising new anti-ischemic agent which may be useful in improving functional recovery following heart surgery to correct congenital heart defect in infants. I would like to suggest that further studies of these agents be vigorously pursued with the aim of obtaining better and more specific AMPK inhibitors. If AMPK inhibitors improve recovery of heart function following by-pass surgery in clinical studies as observed in these pre-clinical studies, AMPK inhibitors may be valuable new addition to the growing list of pharmacological agents currently available for the treatment of angina and other forms of ischemic heart disease.

Conclusion:

In conclusion, AMPK plays an important role in the maturation and regulation of fatty acid oxidation in newborn rabbit hearts.

Future directions:

1) With the use of recombinant DNA technology, it should be relatively easy to produce an AMPK knockout mouse. An adequate perfusion system for mouse hearts now exists in which fatty acid oxidation, AMPK, ACC activity and levels of malonyl CoA could be measured directly. If these were done in AMPK knockout mice results of such studies would further clarify our understanding of the role of AMPK in the regulation of fatty acid oxidation in newborn hearts.
2) AMPK is present prior to birth in 29-day gestation fetal rabbit hearts. It is not clear what role AMPK plays in the regulation of carbohydrate or fatty acid metabolism in the fetal heart. Certainly the fetal heart is more reliant on carbohydrate metabolism for ATP production than the newborn heart. Interestingly, AMPK has recently been implicated in the regulation of glucose utilization and glycogen turnover. It would be worthwhile to determine the role of AMPK in the regulation of both carbohydrate and fatty acid metabolism in fetal rabbit hearts.
3) Accumulating evidence from studies in newborn rabbit hearts and adult rat hearts strongly suggest that AMPK regulates fatty acid oxidation in the heart. However, it still remains unclear whether AMPK regulates fatty acid oxidation only in healthy heart tissue or whether the same regulation exists in diseased hearts. One possible approach to investigate this is to determine the effect of AMPK inhibitors on indices of mechanical function and fatty acid oxidation in diabetic hearts where excessive fatty acid oxidation has been associated with depressed cardiac performance. I would postulate that AMPK inhibitors will decrease the overt reliance of diabetic heart on fatty acid oxidation as a main source of ATP and improve cardiac function.

4) The result from my perfusion of newborn rabbit heart with AMPK inhibitors suggest a need to further explore the cardioprotective effects of these agents. A collaborative study with a medicinal chemist may be necessary to make slight structural modifications to the iodotubercidin chemical structure with the hope of synthesizing more specific AMPK inhibitors. It would be interesting to have a panel of such putative AMPK inhibitors and to test their effects on fatty acid oxidation and AMPK activity in both aerobic and reperfused-ischemic hearts. It is possible that one of these newer AMPK inhibitors may become a new drug available for the treatment of ischemic heart disease.

5) Both α1 and α2 isozymes of AMPK catalytic subunit are expressed in newborn rabbit heart. The contribution of each isozyme to total AMPK activity, however, remains to be determined. Moreover, it is unclear which of these isozymes is more important in the overall regulation of fatty acid oxidation in newborn rabbit hearts. Differential regulation of AMPK α1 and α2 isozymes has been proposed by Hardie's group (Salt et al., 1998), efforts to develop a selective AMPK isozyme inhibitor may better clarify the mechanism by which AMPK isozymes regulate myocardial fatty acid oxidation in the newborn heart as well as facilitate selective targeting of AMPK.

6) It is of interest that a monoclonal antibody raised against rat heart ACC does not recognize newborn rabbit heart ACC. For this reason, streptavidin-conjugated horseradish peroxidase was used to visualize ACC in results presented in this thesis. It would be desirable to clone newborn rabbit heart ACC and delineate potential phosphorylation sites for both AMPK and PKA. This would further our understanding of tissue specific ACC regulation and the possible implications for regulation of fatty acid oxidation in the newborn heart.

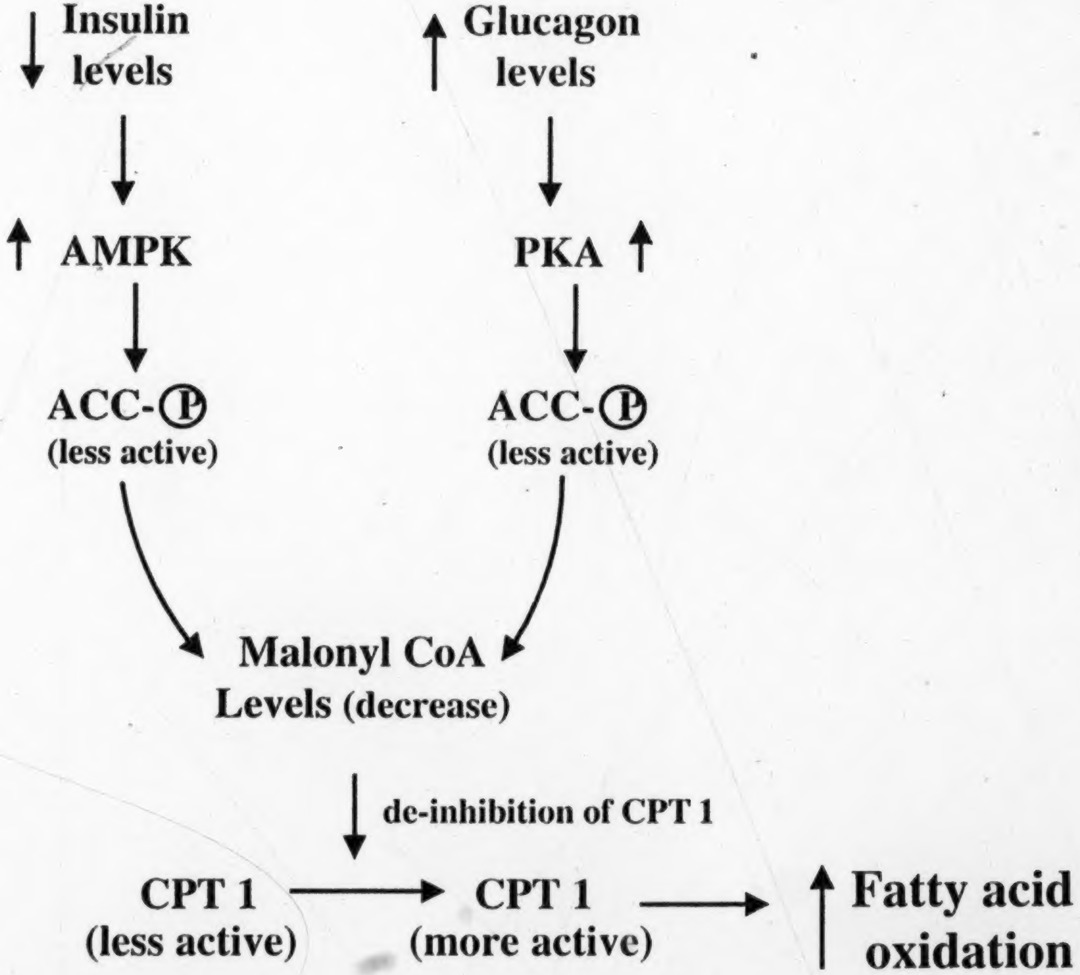
7) AMPK kinase is present in the newborn rabbit heart. However, this kinase kinase remains to be fully characterized. Identification and characterization of this kinase would further our understanding of the mechanism by which AMPK itself is regulated in newborn rabbit hearts. It will also help us understand whether or not fatty acid oxidation could be regulated via modulation of AMPK kinase activity.
8) Malonyl CoA decarboxylase (MCD) exists in the rat heart. MCD may regulate myocardial fatty acid oxidation via regulation of levels of malonyl CoA. Characterization of MCD in the newborn heart is a worthwhile project. This may lead to the development of novel pharmaceuticals that alter fatty acid oxidation.

If results of these potential experiments turn out to be positively in favor of my central hypothesis, then AMPK may indeed be a key target for modulating myocardial fatty acid oxidation (both in experimental animals and humans) in health or disease.

Figure 8.1

Overview of the proposed mechanism by which fatty acid oxidation matures in the newborn rabbit heart.

The decrease in circulating levels of insulin following birth result in activation of AMPK. Increased AMPK activity results in phosphorylation and inactivation of ACC. Myocardial malonyl CoA levels decrease thereby resulting in de-inhibition of CPT 1, the rate-limiting enzyme involved in fatty acid uptake into the mitochondria. This results in increased uptake and oxidation of fatty acids. Similarly the rise in glucagon levels in the immediate post-natal period results in increased cAMP levels and an activation of PKA that phosphorylates and inactivates ACC. The resultant decrease in ACC activity and malonyl CoA levels leads to an increase in fatty acid oxidation in newborn heart.


↓ Insulin levels
↑ Glucagon levels
↑ AMPK
PKA ↑
ACC-Ⓟ (less active)
ACC-Ⓟ (less active)
Malonyl CoA Levels (decrease)
de-inhibition of CPT 1
CPT 1 (less active)
CPT 1 (more active)
↑ Fatty acid oxidation

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